Phosphatase inhibitors influence proteolytic cleavage pattern of glycine transporter GlyT2 N-terminus

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Calpain activation in spinal cord neurons results in removal of the glycine transporter N-terminal region. In this work we have compared the cleavage patterns of released peptides in presence of both okadaic acid and sodium orthovanadate. Under basal calcium concentration the calpain cleavage patterns are significantly different for these two phosphatase inhibitors. In contrast to one immunopositive peptide band present in presence of sodium orthovanadate, two additional bands are observed in presence of okadaic acid. This indicates that phosphorylation might block/activate certain proteolytic cleavage sites on GlyT2 N-terminus *in vivo*.

Key words: glycine, transporter, GlyT2, calpain, okadaic acid, orthovanadate, phosphatase, calcineurin.

Glycine transporter GlyT2 belongs to the family of sodium dependent neurotransmitter transporters, proteins that maintain the clearance of several important neurotransmitters (NELSON, 1998; JURSKY & BALIOVA, 2002). One of them, glycine, is the major inhibitory neurotransmitter in the hindbrain and spinal cord (LEGENDRE, 2001) as well as important neuromodulator in all brain parts (BERGER et al., 1998; BREGERON et al., 1998). Many proteins in living cells are modified by calpain protease under pathological, but also in normal cell conditions (GOLL et al., 2003). Calpain cleavage sites in polypeptide chains are not frequent and usually are located at interdomain boundaries (DAYTON et al., 1975; PATTNI et al., 2003). In our previous work we found that activation of calpain protease in spinal cord neuronal cultures or synaptosomal preparation leads to truncation of extended N-terminal domain of glycine transporter GlyT2 (BALIOVA et al., 2004; BALIOVA & JURSKY, 2004). In conditions when synaptosomes were lysed in high calcium hypoosmotic buffer almost all transporters have been truncated in 15 minutes at 37 °C by endogenous calpain activity present in the spinal cord. However, in native conditions when neurons and synaptosomes remained intact and calpain protease was activated by addition of calcium ionophore, only a small fraction of transporters has been truncated. Surface biotinylation experiments showed that the majority of transporters truncated under intact conditions are located on the cellular surface. There are two explanations for this observation. One comes from previous re-

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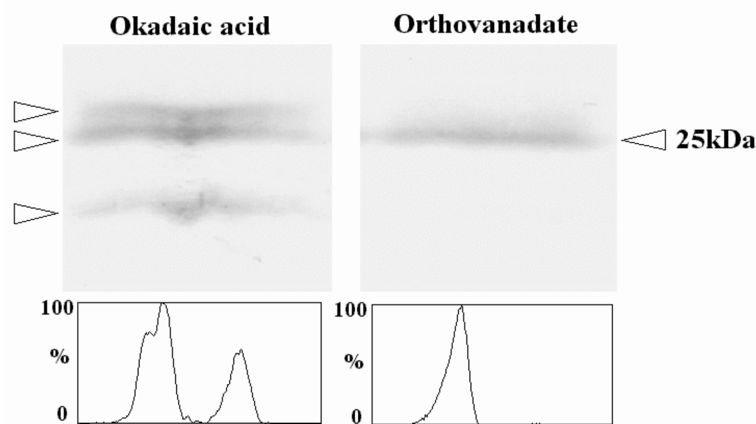

Fig. 1. Proteolytic cleavage pattern of glycine transporter GlyT2 N-terminus in presence of phosphatase inhibitors. Fresh spinal cord tissue has been homogenized in the presence of each inhibitor (freshly prepared final 1 μM okadaic acid, 200 μM sodium orthovanadate) in 0.32 M sucrose/10mM Hepes-NaOH, pH 7.4. Post nuclear supernatant was obtained with 10 min centrifugation at 1,000 *g*. Spinal cord synaptosomes were obtained after 20 min centrifugation at 12,000 *g*. Synaptosomes were washed with 25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and lysed in 25mM Hepes/NaOH pH 7.4 for 15 minutes at 37 ℃. All treatments described above were done in presence of phosphatase inhibitors. Aliquot of each sample was dissolved in SDS sample buffer and resolved on 12% PAG. Gel was transferred to immobilon and immunoblotted with antibodies against the N-terminal domain of glycine transporter GlyT2. Antibodies were affinity purified on recombinant GlyT2 N-terminal protein. Relative quantification of band intensity was made by Scion Image software. (Arrows indicate position of released bands and position of 25 kDa protein standard-Broad Range standard from New England Biolabs.)

ports that calpain is usually active at the cellular membranes and its activation is connected with its translocation to the membrane (Molinari et al., 1994; Michetti at al., 1996). The second reason for resistance of intracellular pool of transporters to calpain could be its phosphorylation. Number of cases has been reported where phosphorylation of proteins inhibits or activates their cleavage by calpain (Bi et al., 2000; Rong et al., 2001; Nicolas et al., 2002). There is at least one report about dopamine transporter showing the phosphorylation of its N-terminal domain (Foster at al., 2002). The assumption that cytosolic regions of neurotransmitter transporters located in intracellular pool are phosphorylated is supported also by fact that stimulation of protein kinases downregulates transporters from the cellular membrane (Bauman et al., 2000). Extended domain of glycine transporter GlyT2 contains several potential phosphorylation sites (Liu et al., 1993). We were therefore interested if phosphorylation of N-terminal domain could influence its sensitivity to calpain cleavage. When 5 mM calcium was added to spinal cord synaptosomes under conditions of hypoosmotic lysis, the N-terminal sequences were in majority released as 25 kDa protein fragments corresponding to major part of the transporter N-terminus (M. Baliova & F. Jursky, unpublished results). Under these conditions the N-terminal region of GlyT2 is likely quantitatively dephosphorylated by abundant calcium/calmodulin dependent phosphatase calcineurin. Our attempt to inhibit calcineurin with cyclosporine did not caused changes in calpain cleavage patterns. Cyclosporine however acts as inhibitor only after its interaction with cellular component cyclophilin (Marks, 1996). In addition calpain cleaves the calcineurin, which makes it constitutively active and it is not clear if modified enzyme is still inhibited by cyclosporine (Wu et al., 2004). When experiments were done without adding the external calcium, limited cleavage of GlyT2 N-terminal domain still could be observed. We explain this by activation of small fraction of neuronal calpain by extracellular calcium entrapped during the homogenisation. In these conditions we observed significant difference in cleavage patterns with two phosphatase inhibitors. Okadaic acid inhibits mostly serine/threonine phosphatase, while orthovanadate inhibits tyrosine phosphatases. Figure 1 shows that in the presence of orthovanadate single peptide fragment is released which is rec-

ognized by anti-GlyT2N-terminal antibodies. In presence of okadaic acid two additional fragments are released and recognized with the same antibodies. We cannot fully explain this or predict biological consequences of the above observation. Results however indicate that certain proteolytic cleavage sites in the N-terminal region of glycine transporter GlyT2 might be influenced by phosphorylation.

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