Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A

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Abstract In this study we have examined the effect of the SNARE protein syntaxin 1A on the glycine transporters GLYT1 and GLYT2. Our results demonstrate a functional and physical interaction between both glycine transporters and syntaxin 1A. Co-transfection of syntaxin 1A with GLYT1 or GLYT2 in COS cells resulted in approximately 40% inhibition in glycine transport. This inhibition was reversed by the syntaxin 1A-binding protein, Munc18. Furthermore, immunoprecipitation studies showed a physical interaction between syntaxin 1A and both transporters in COS cells and in rat brain tissue. Finally, we conclude that this physical interaction resulted in a partial removal of the glycine transporters from the plasma membrane as demonstrated by biotinylation studies.

Key words: Glycine transport; Neurotransmitter reuptake; Syntaxin; SNARE protein; Protein regulation; Membrane trafficking

1. Introduction

Glycine is an inhibitory neurotransmitter mainly present in the spinal cord and the brain stem, where it plays a role in a variety of motor and sensory functions. Moreover, glycine modulates the action of glutamate on the *N*-methyl-D-aspartate receptors. After the release of glycine and its binding to postsynaptic receptors, high affinity reuptake mechanisms play an important role in the removal of glycine from the extracellular space, thereby terminating the neurotransmission signal. The fine-tuning of this reuptake mechanism is thought to control the duration of the neurotransmission signal and therefore the overall synaptic action.

Reuptake of glycine from the intersynaptic space is modulated by high affinity glycine transporters. Two glycine transporters have been cloned: GLYT1 and GLYT2. The cDNAs encoding GLYT1 and GLYT2 have been used for extensive research on these transporters. The subjects of these studies have been *N*-glycosylation, localization, and structure–function relationships [1,2]. Concerning the important role neurotransmitter transporters have in neurotransmission it seems obvious that they must be properly regulated. However, not much is known about the regulation of the glycine transporters. Previously it was found that activation of protein kinase C (PKC) by phorbol esters resulted in an inhibition of glycine transport [3]. So far, this has been the only regulation mechanism described for the glycine transporters.

Syntaxin 1A is a plasma membrane protein from the SNARE system which regulates intracellular membrane trafficking [4]. Furthermore, it has a role in the regulation of several ion channels (e.g. Ca^{2+} and the CFTR Cl^- channels, [5] and [6], respectively) and the γ-aminobutyric acid (GABA) transporter GAT1 [7]. For this GABA transporter, which belongs to the same family of sodium-dependent neurotransmitter transporters as the glycine transporters, it was found that it interacts directly with syntaxin 1A and that this interaction is regulated by PKC [7].

In our ongoing research on the function and regulation of the glycine transporters we became interested in the regulation of glycine transporters by SNARE proteins. Here we report that syntaxin 1A regulates and interacts directly with the neuronal glycine transporters GLYT1 and GLYT2.

2. Materials and methods

2.1. Materials

COS-7 cells were from American Type Culture Collection (Manassas, VA, USA). Antibodies against syntaxin 1A, SNAP-25 and Munc18 were obtained from Upstate Biotechnology (Lake Placid, NY, USA), Stressgen (Victoria, Canada) and Transduction Laboratories (Lexington, KY, USA), respectively. GLYT1 and GLYT2 antibodies have been characterized [8].

2.2. cDNA constructs

The cDNAs encoding syntaxin 1A, Munc18a and SNAP-25 were PCR amplified from a rat brain cDNA library using the following PCR primers: ccgaattccatgaaggaccgaacccaggagctccgc and gggaattcctatccaaagatgcccccgatggtggagg for syntaxin 1A; cgaattccgccatggccccccattggcctc and tgaattcgggcagctgattttaactgcttatttcttcg for Munc18a; cacgaattccatggccgaggacgcagacatgcg and aggcttaagttaaccacttcccagcatctttg for SNAP-25. The PCR products were cloned into pcDNA3 (Invitrogen), and sequenced completely. The cDNA encoding syntaxin 1A was cloned into pcDNA3 in both the sense and antisense directions. The cDNA constructs of GLYT1b and GLYT2a in pcDNA3 have been described [9].

2.3. Cell transfection

Transfection of COS cells was carried out using LipofectAMINE (Life Technologies) in serum free Dulbecco's modified Eagle's medium (DMEM). Reproducible results were obtained when transfections were performed with 50% confluent cells on a 60 mm petri dish using 2 μg of total DNA. The total DNA consisted of 0.5 μg of each cDNA construct completed with pcDNA3 plasmid up to 2 μg. The lipid/DNA mixture was incubated with the cells for 3 h. Subsequently cells were rinsed and re-fed with DMEM complemented with 10% fetal calf serum. Forty-eight hours after transfection cells were assayed for glycine uptake.

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Abbreviations: GLYT, glycine transporter; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction

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2.4. Uptake assays

Transport assays in transfected COS cells were performed at 37°C in HEPES-buffered saline as described [2]. All incubations were performed in triplicate using 10 μM radiolabeled glycine (DuPont). The incubation period was always 6 min. Protein concentrations were determined by the method of Bradford [10]. Where indicated, the transfected cells were preincubated for 1 h at 37°C in HEPES-buffered saline in the presence or absence of 1 mM glycine.

2.5. Immunoblots and immunoprecipitation

SDS–PAGE and Western blotting have been described [2]. Co-immunoprecipitation using a protein extract from transfected COS cells or from rat brain tissue was done in the following lysis buffer: 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride. Where indicated, 1 mM glycine was added to the lysis buffer. The protein extract from whole rat brain was obtained using a glass homogenizer. Cell debris was removed by centrifugation. 50 μg of protein was incubated with GLYT1 or GLYT2 antibodies for 3 h in 1 ml lysis buffer, followed by the addition of 30 μl of a 50% slurry of protein A-Sepharose beads. After an additional 1 h incubation, the beads were washed three times with 1 ml lysis buffer. The proteins were then eluted from the beads with Laemmli sample buffer and subjected to Western blotting with the indicated antibody.

2.6. Cell surface labeling

Cell surface proteins of transfected COS cells were labeled with the cell membrane impermeable reagent sulfo-*N*-succinimidyl-6-biotinamido hexanoate as described [11]. In brief, cells were washed with phosphate-buffered saline (PBS) and incubated for 30 min on ice in the presence of 1.5 mg/ml sulfo-*N*-succinimidyl-6-biotinamido hexanoate in PBS. Then, cells were washed, and the excess of reagent was quenched with 10 mM lysine in PBS for 10 min. Cells were lysed in immunoprecipitation buffer (150 mM NaCl, 50 mM HEPES–Tris, 5 mM EDTA, 1% Triton X-100, 0.25% deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4), and biotinylated proteins were precipitated with agarose–streptavidin. Precipitated proteins were fractionated by SDS–PAGE and analyzed by Western blotting. Quantitation of protein bands was performed on a densitometer model 300A in combination with Image Quant software (Molecular Dynamics).

3. Results

3.1. Syntaxin 1A functionally regulates GLYT1 and GLYT2 by a direct physical interaction

To test the hypothesis that syntaxin 1A functionally regulates glycine transporters, glycine transport was assayed in COS cells expressing GLYT1 or GLYT2 with or without co-expression of syntaxin 1A. Both glycine transporters and the SNARE proteins used in this study (syntaxin 1A, SNAP-25 and Munc18) were expressed well in these cells, as assessed from immunoblots with specific antibodies. Moreover, no endogenous signals for these proteins were observed in untransfected cells (data not shown). We chose to assay glycine transport at 10 μM glycine because at this concentration the non-specific transport of glycine is low compared to specific transport. At 10 μM glycine, specific transport is typically 5–10 times higher in transfected COS cells compared to untransfected cells. This may be explained by the differences in K_m value between specific and non-specific glycine transport; the K_m value for non-specific transport is around 10 times higher than specific transport in COS cells (unpublished data). Expression of syntaxin 1A never affected non-specific glycine transport in COS cells, which was always subtracted from the specific transport. We observed a substantial inhibition (around 40%) of glycine transport in both transporter isoforms (GLYT1 and GLYT2) when syntaxin 1A was co-expressed (Fig. 1A,B). This inhibition was not observed when an antisense construct of syntaxin 1A was used for transfection (data not shown).

To show that syntaxin 1A did not alter the expression of the glycine transporters, we extracted in each experiment total GLYT1 and GLYT2 proteins from the transfected COS cells and performed Western blot analysis. Typical results are shown in Fig. 1C,D. As has been described previously [8], glycine transporters transiently expressed in COS cells show a non-mature protein band, and a fully glycosylated mature form which immunoreacts with either GLYT1 or GLYT2 antibodies. The fully processed transporters are shown in Fig. 1C,D, indicating that neither transfection levels nor glycine transporter expression seem to be changed by syntaxin 1A co-expression.

The inhibition of glycine transport by syntaxin 1A was reversed by co-expression of the syntaxin-binding protein Munc18 (Fig. 1A,B). Munc18 binds tightly to syntaxin 1A and in this manner prevents the binding of syntaxin 1A to other binding partners [12]. Probably this also holds for the glycine transporters, resulting in a rescuing of the syntaxin 1A inhibition. This result additionally proves that the inhibitory effect of syntaxin 1A is not due to translational competition causing the reduction in glycine transport.

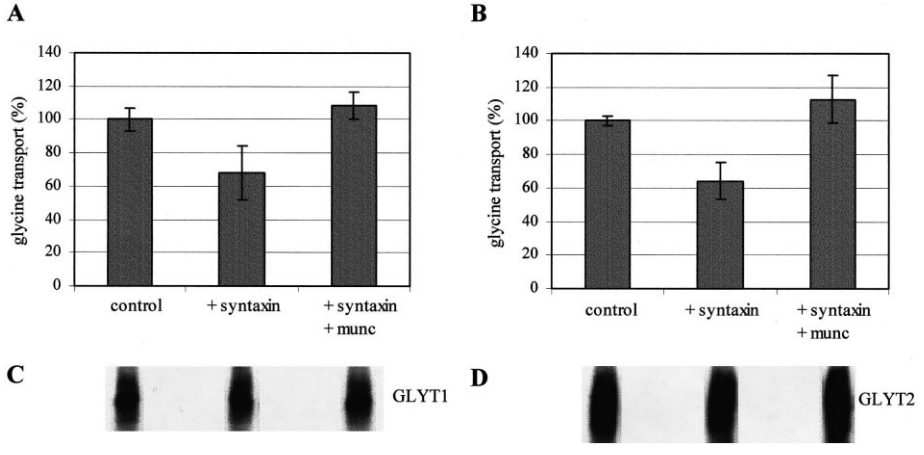

Fig. 1. The glycine transporters GLYT1 (A, $n=7$) and GLYT2 (B, $n=6$) are functionally inhibited by syntaxin 1A. This inhibition is reversed by co-expression of the syntaxin 1A-binding protein Munc18. Immunoblots with specific antibodies against GLYT1 (C) and GLYT2 (D) demonstrate that syntaxin 1A does not affect GLYT1 or GLYT2 expression. The bands shown are from the mature glycosylated GLYT1 and GLYT2 proteins.

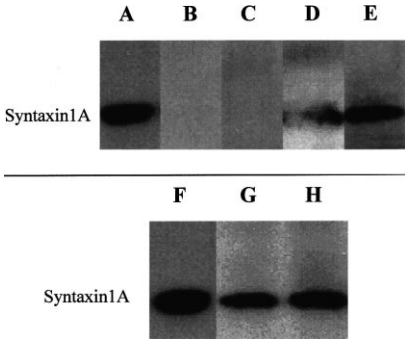

Fig. 2. Co-immunoprecipitations demonstrate that physical interactions exist between GLYT1 and GLYT2 with syntaxin 1A in transfected COS cells and in rat brain tissue. Immunoblots stained with Ig-syntaxin 1A: protein extract of COS cells expressing syntaxin 1A (A), COS cells expressing syntaxin 1A were immunoprecipitated with Ig-GLYT1 (B), as B using Ig-GLYT2 for immunoprecipitation (C), COS cells expressing syntaxin 1A together with GLYT1 were immunoprecipitated with Ig-GLYT1 (D), COS cells expressing syntaxin 1A together with GLYT2 were immunoprecipitated with Ig-GLYT2 (E), protein extract of rat brain (F), rat brain extract immunoprecipitated with Ig-GLYT1 (G), rat brain extract immunoprecipitated with Ig-GLYT2 (H).

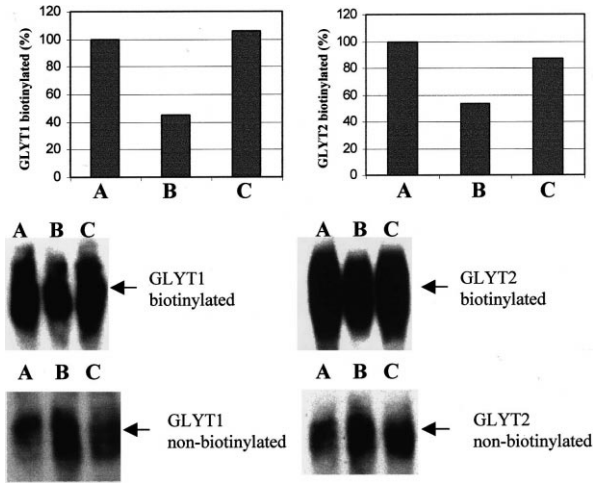

Fig. 3. Cell surface biotinylation shows that syntaxin 1A controls the number of glycine transporters on the plasma membrane in transfected COS cells. This effect is reversed by Munc18. A: Glycine transporter. B: Glycine transporter co-expressed with syntaxin 1A. C: Glycine transporter co-expressed with syntaxin 1A and Munc18. Quantitation of surface GLYT1 and GLYT2 as percentage of total protein is shown in the graphs.

The functional data suggested an interaction between syntaxin 1A and GLYT1 and GLYT2. To test this hypothesis we performed co-immunoprecipitation studies in which we used COS cells transfected with syntaxin 1A together with GLYT1 or GLYT2. We also used COS cells transfected only with syntaxin 1A as a control. The results of these studies, in which we used GLYT1 or GLYT2 antibodies for immunoprecipitation and syntaxin 1A antibodies in the subsequent Western analysis, are shown in Fig. 2A–E. Both GLYT1 and GLYT2 antibodies co-immunoprecipitate syntaxin 1A, but not in COS cells transfected only with syntaxin 1A. These results demonstrate that a physical interaction exists between the glycine transporters and syntaxin 1A in transfected COS cells. To test if this physical interaction also exists in brain tissue, we performed co-immunoprecipitation using a protein extract from rat brain tissue under the same conditions. The results show that the same physical interactions exist in this tissue, additionally indicating a possible physiological role for the interaction (Fig. 2F–H).

3.2. Syntaxin 1A controls the number of glycine transporters on the plasma membrane

The inhibitory effect of syntaxin 1A could result from an effect on the glycine transporter (e.g. conformational changes) or from a reduction in the number of plasma membrane-resident glycine transporters. To address this issue we performed two different types of experiments: kinetic analysis and surface biotinylation studies.

The kinetic parameters K_m and V_{max} for both glycine transporters in the presence and absence of syntaxin 1A are presented in Table 1. Although a small change in the K_m value was detected for GLYT1, co-expression with syntaxin 1A mainly resulted in a lower V_{max} value for both transporters. This change in maximum velocity of transport might be explained by a change in the number of transporter-binding sites present on the plasma membrane. Surface biotinylation studies revealed changes in surface GLYT1 and GLYT2 immunoreactivities (Fig. 3). Both transporters were less present on the cell surface when co-expressed with syntaxin 1A. Importantly, this effect was reversed by Munc18. On the other hand, the non-biotinylated but fully processed (internal) transporters were more abundant when syntaxin 1A was co-expressed with GLYT1 or GLYT2.

Table 1
Kinetic data of the glycine transporters GLYT1 and GLYT2 in the presence and absence of syntaxin 1A

Type of cDNA transfection	K_m (μM)	V_{max} (pmol mg^{-1} 6 min^{-1})
GLYT1	85.0 ± 3.0	26.4 ± 0.8
GLYT1+syntaxin 1A	65.0 ± 1.3	14.2 ± 0.3
GLYT2	75.1 ± 16.5	3.6 ± 0.7
GLYT2+syntaxin 1A	82.9 ± 13.3	2.3 ± 0.3

The results from both the biotinylation and the kinetic experiments show that syntaxin 1A inhibits glycine transport by interfering with the delivery and/or the removal of GLYT1 and GLYT2 to and/or from the plasma membrane.

3.3. Glycine and SNAP-25 do not alter the syntaxin–transporter interaction

L-type, N-type and P/Q-type Ca^{2+} channels are functionally regulated by the SNARE proteins syntaxin 1A and SNAP-25 [13,14]. So we became interested to see if the glycine transporters also interacted with SNAP-25. For this, we co-transfected COS cells either with GLYT1 or GLYT2, together with SNAP-25, or with the combination syntaxin 1A and SNAP-25. Subsequently, glycine transport was measured in these cells. Our results indicated that SNAP-25 had no functional effect on transport when expressed alone and no additional effect in combination with syntaxin was detected (data not shown). Furthermore, we were not able to co-immunoprecipitate SNAP-25 using Ig-GLYT1 and Ig-GLYT2 in transfected COS cells or in rat brain extract (data not shown). So SNAP-25 seems not to have a direct interaction with the glycine transporters.

The GABA transporter was found to be regulated by extracellular GABA, and it was suggested that this regulation

might be due to an alteration in the interaction of the transporter with syntaxin 1A [15]. To test if glycine alters the interaction between the glycine transporters and syntaxin 1A we performed co-immunoprecipitation studies using rat brain tissue in the presence and absence of 1 mM glycine. We did not observe any differences between the co-immunoprecipitations for GLYT1 and GLYT2 with syntaxin 1A in the presence or absence of glycine (data not shown). We also observed no differences in the inhibition of both glycine transporters caused by syntaxin 1A when transfected COS cells were preincubated for 1 h with or without 1 mM glycine (data not shown). These results indicate that glycine does not alter the interaction of syntaxin 1A with the glycine transporters under the conditions used.

4. Discussion

We used COS cells as an expression system to explore the role of the SNARE protein syntaxin 1A on the glycine transporters GLYT1 and GLYT2. The principal findings of the studies include: (1) both glycine transporters can be functionally modulated by syntaxin 1A, (2) the glycine transporters physically interact with syntaxin 1A both in transfected cells and in brain tissue, (3) syntaxin 1A decreases the number of GLYT1 and GLYT2 proteins on the plasma membrane, but does not affect the overall expression of glycine transporters, (4) Munc18 reverses the inhibition caused by syntaxin, (5) SNAP-25 seems not to participate in the regulation, and (6) glycine does not change the interaction between syntaxin 1A and the glycine transporters.

Syntaxin 1A is a SNARE protein involved in the intracellular membrane trafficking pathways [4]. Our results show that co-expression of syntaxin 1A with both glycine transporters results in fewer transporter proteins on the plasma membrane as compared to control cells not expressing syntaxin 1A. Most likely, syntaxin 1A somehow interferes with the trafficking of the transporters to and/or from the plasma membrane. Although the exact mechanism by which syntaxin acts is yet unknown, it seems reasonable to think that syntaxin 1A controls the number of glycine transporter molecules on the plasma membrane by a direct physical contact, regulating in this way neurotransmitter transport. Future studies should clarify how syntaxin plays its role, and how syntaxin itself is regulated in order to function in both exocytosis and neurotransmitter reuptake.

The glycine transporters GLYT1 and GLYT2 share respectively 41 and 48% homology with the GABA transporter. Nevertheless, these transporters behave very similarly in their interaction with syntaxin 1A. The GABA transporter was also found to interact directly with syntaxin 1A resulting in an inhibition of GABA transport [16]. Moreover, PKC is involved in this interaction [16], and modulates a re-localization of the transporter from intracellular pools to the plasma membrane [7]. Concerning the growing number of membrane proteins which have interaction with syntaxin 1A, it becomes more and more obvious that syntaxin 1A acts as a general regulator in plasma membrane protein trafficking, something that was mentioned before [17].

Syntaxin 1A is broadly distributed throughout the central and peripheral nervous systems on synaptic terminal membranes [18]. GLYT2 is present in the spinal cord, brain stem and cerebellum, whereas GLYT1 has a broader distribution [8]. The co-localization together with the data presented here opens new possibilities for studying the regulation of the glycine transporters.

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