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Group-selective reagent modification of the sodium- and chloride-coupled glycine transporter under native and reconstituted conditions

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Glycine transporter from rat brain stem and spinal cord is inactivated by specific sulfhydryl reagents. Modification of lysine residues also promotes a decrease of the transporter activity but in a lesser extent than that promoted by thiol group reagents. Mercurials showed a more marked inhibitory effect than maleimide derivatives. SH groups display a similar reactivity for *p*-chloromercuribenzenesulfonate (pCMBS) and mersalyl in synaptosomal membrane vesicles and proteoliposomes reconstituted with the solubilized transporter. However, different reactivity is observed with *N*-ethylmaleimide (MalNEt), the greatest effect being attained in membrane vesicles. The rate of inactivation by pCMBS and MalNEt is pseudo-first-order showing time- and concentration-dependence. pCMBS and MalNEt decrease the V_{max} for glycine transport and to a lesser extent act on the apparent K_m. Treatment with dithiothreitol (DTT) of the transporter modified by pCMBS results in a complete restoration of transporter activity indicating that the effect exercised by the reagent is specific for cysteine residues on the protein. It is concluded that SH groups are involved in the glycine transporter function and that these critical residues are mostly located in a relatively hydrophilic environment of the protein.

Introduction

Response to neurotransmitters at postsynaptic receptors depends upon both the concentration of neurotransmitter reached in the synaptic cleft and the duration that such concentrations are maintained. For neurons to maintain rapid and efficient chemical communication with effector sites, neurotransmitters must have a brief extracellular lifetime, paralleling the rise and fall of presynaptic excitation. During the past few years it has became clear that high-affinity amino acid uptake into presynaptic terminals or neighbouring glial elements serves to terminate the overall process of synaptic transmission [1–7]. These transporters are sodium ion/neurotransmitter cotransporters and are able to accumulate the neurotransmitter against considerable concentration gradients by using the electrochemical gradient of sodium ions.

In sharp constrast to the detail with which other proteins involved in signal transduction are understood and despite a wealth of bioenergetic and kinetic studies on transport itself [8,9], our understanding of the molecular principles guiding neurotransmitter uptake is considerably limited. Due, perhaps, to their low abundance and poor stability in vitro [10], purification strategies as yet yielded little structural data. Only within the past few years has a Na^+-dependent GABA transporter from rat brain been purified [11]. One of the important transporters in the spinal cord and in the brain stem of vertebrates is the one for glycine. This system catalyzes coupled electrogenic transport of sodium, chloride and glycine at a stoichiometry of $2Na^+$ and $1Cl^-$/glycine zwitterion [12–14]. Recently, the glycine transporter has been isolated, partially purified and the transport activity reconstituted into liposomes [15,16]. However, little is known about the residues involved in the catalytic activity of the trans-

Abbreviations: pCMBS, *p*-chloromercuribenzenesulfonate; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GABA, 4-aminobutyric acid: MalNEt, *N*-ethylmaleimide; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonate.

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porter. A better understanding of the mechanisms of action requires the identification of the functional groups of the protein implicated in the translocation cycle.

In this report, we have used a chemical modification approach of glycine transporter in membrane vesicles derived from the synaptic plasma membrane and in reconstituted proteoliposomes. The results presented here show that sulfhydryl groups play an important role in the activity of the glycine transporter. In membrane vesicles and proteoliposomes SH groups involved in the glycine transporter function are accessible to membrane-impermeant SH reagents such as mersalyl and pCMBS, and thus are most likely located at the outer, water-accessible surface of the protein. As well, hydrophobic SH reagents such as several maleimide derivatives inhibit glycine transport but in a minor and different extent in membrane vesicles and proteoliposomes. Similar differential reactivity was observed with other reagents. It could be attributed to differences in the membrane environment in both preparations, which provoques dissimilar accessibility of the amino acid groups to the chemical modifiers.

Materials and Methods

Materials

[2-^{3}H]Glycine (1757.5 TBq/mol) was obtained from New England Nuclear, Boston, MA, U.S.A. Soybean phospholipids (asolectin), Associated Concentrates, New York, U.S.A., were partially purified as described [17]. Crude lipids were extracted from bovine brain [18]. Sephadex G-50 was from Pharmacia, Uppsala, Sweden. Dithiothreitol, mersalyl, *p*-chloromercuribenzene sulfonate, fluorescein isothiocyanate, diethylpyrocarbonate, *N*-ethylmaleimide and cholic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cholic acid was recrystallized [17] and neutralised with NaOH to pH 7.4. Phenylglyoxal, trinitrobenzenesulfonic acid and *N*-acetylimidazole from Fluka Chemie AG, Buchs, Switzerland, and 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid from Molecular Probes, Eugene, OR, U.S.A. Nitrocellulose filters used for the transport assays were Millipore type HAWP (0.45 μm). All other reagents were of the highest purity available. Milli-Q water was used in the preparation of all aqueous solutions.

Methods

Membrane vesicle preparation

Adult male rats of the Wistar strain (150–200 g) were used. Ficoll-gradient-purified synaptosomes from rat brain stem and spinal cord were prepared as described [12,19].

The membrane vesicles were washed in a medium containing 145 mM potassium gluconate, 10 mM Hepes-Tris (pH 7.4), 1 mM $MgSO_4$, 0.5 mM EDTA, 1% (by vol.) glycerol (buffer A) and resuspended in the same medium at a protein concentration of 15–25 mg/ml. The membranes were quick-frozen in liquid nitrogen and stored at −70°C until use.

Reconstitution of solubilized membrane proteins

The sodium and chloride-coupled high affinity glycine transport activity was solubilized from rat brain membranes and reconstituted into liposomes as described [15,16]. Liposomes were prepared as follows: a stock chloroform mixture of asolectin and rat brain lipids (85 : 15, mol%) was dried under a stream of N_2 at 25°C in a round-bottom flask followed by resuspension in freshly distilled diethyl ether and subsequent drying under N_2. The thin film of lipids was then resuspended in buffer A. The suspension was sonicated in a bath type sonicator until a clear solution was obtained. This phospholipid suspension was mixed with the fractions in the presence of 0.6 M NaCl and 1.5% (w/v) sodium cholate (final concentrations). After 15 min on ice, the reconstitution of the glycine transporter into liposomes was performed by removing the detergent by passing the former reconstitution mixture (up to 200 μl), through a 1ml minicolumn containing Sephadex G-50–80 preswollen in the desired 'in' medium (which was usually the same as that used for resuspension of liposomes, see above), and then centrifuged (approx. 1500 × *g* for 2.5 min). Before use, the syringes were centrifuged (as above) in order to dry the gel.

Procedures of chemical modification

Membrane vesicles were suspended at a protein concentration of 4–5 mg/ml in buffer A and incubated in the presence of the required concentrations of different protein-modifying reagents, added from fresh concentrated solutions. Phenylglyoxal and diethylpyrocarbonate solutions were made in ethanol; those of fluorescein isothiocyanate and maleimidylanilinonaphthalene sulfonic acid (MIANS) in dimethylformamide and those of *N*-acetylimidazole, trinitrobenzenesulfonate, MalNEt, mersalyl and pCMBS in buffer A. The following incubation conditions were used to achieve modification specificity [20–22]: maleimide derivatives and *N*-acetylimidazole at room temperature; pCMBS and mersalyl, room temperature in the dark; phenylglyoxal, 30°C; trinitrobenzenesulfonate 30°C, the pH of buffer A being changed to 8.8; fluorescein isothiocyanate, 30°C, pH 8.8 in the dark; diethylpyrocarbonate, 4°C, pH 6.2. The mixtures were incubated with gentle shaking for 10 min. The reactions were terminated by addition of 10 volumes of ice-cold buffer A. The excess of reagent was removed by centrifugation at 27000 × *g* for 15 min. Membrane vesicles were resus-

pended in the same buffer and the centrifugation-resuspension process was repeated three times.

Other chemical modification experiments were performed with the solubilized glycine transporter reconstituted into liposomes (proteoliposomes). Proteoliposomes (lipid/protein, 35:1) were incubated with the reagents in buffer A as described above. The reactions were terminated by rapidly separating proteoliposomes from the excess reagent by a centrifugation of Sephadex G-50 minicolumns preequilibrated with buffer A (approx. $1500 \times g$ for 2.5 min).

Glycine transport assay

Glycine transport assay was determined in either native or reconstituted preparations as described [12]. Usually, 20 μl of the membrane vesicles (4 mg protein/ml) or proteoliposomes (0.2 mg protein/ml) were incubated at 25°C with 180 μl of an external solution containing [2-^{3}H]glycine (1 μM final concentration, unless otherwise is indicated) in 145 mM NaCl, 10 mM Hepes-Tris (pH 7.4), 1 mM $MgSO_4$, 0.5 mM EDTA, 1% (by vol.) glycerol. All the following steps were performed as described [15]. Experiments shown are typical. The values indicated in the figures represent means obtained for duplicates or triplicates. Each experiment was repeated at least three times with different membrane or proteoliposome preparations. When similar experiments were compared there was a considerable variation in absolute values between different preparations. However, the relative differences within these experiments were highly reproducible, irrespective of the preparation used.

Other methods

Phospholipid phosphate was determined as described [23]. Protein was measured by the method of Bradford [24].

Results and Discussion

The experiment on Table I shows the effect of FITC and TNBS, which show reactivity for the ϵ-amino group of lysine residues of proteins, on the glycine transport. These results indicate that the activity is inhibited by both FITC and TNBS, although dissimilar effect is obtained on plasma membrane vesicles when compared to the reconstituted activity in proteoliposomes. This differential effect found with ϵ-amino group-reagents in both preparations was also observed for the maleimide derivatives, selective reagents for sulfhydryl groups, MalNEt and MIANS. In the same table it is shown that organomercurials such as mersalyl or pCMBS inhibit the glycine transport in a similar extent either in plasma membrane vesicles or in the reconstituted activity in proteoliposomes. Chemical modifying reagents specific for other amino acid residues, such as

TABLE I

Comparative potency of protein-modifying reagents to inhibit glycine transport in membrane vesicles and reconstituted liposomes

Membranes and proteoliposomes were pretreated in the presence of the indicated reagents as described in Materials and Methods. Na^+- and Cl^--dependent uptake of 1 μM [2-^{3}H]glycine was measured in the absence or the presence of the indicated reagents (5–450 nmol/mg of protein, corresponding to 0.02–1 mM). Reagent concentrations required for 50% inhibition of the glycine transport were obtained from the correspondent dose-response curve. 100% of the activity corresponds to 38 pmol Gly/mg protein per 2 min for untreated membranes, and 57 pmol Gly/mg protein per 15 min for untreated proteoliposomes. Values are the means ± S.E. of three replicate experiments. n.d. means that 50% of inhibition is not attained at the concentration range indicated.

Reagent	IC_{50} (nmol/mg protein)	
	membrane vesicles	proteoliposomes
Mersalyl	37.2 ± 2.1	70.5 ± 5.0
pCMBS	32.4 ± 1.7	98.3 ± 5.6
MalNEt	42.0 ± 3.5	n.d.
MIANS	n.d.	225.1 ± 20.3
FITC	n.d.	148.8 ± 7.6
TNBS	252.3 ± 10.4	n.d.

phenylglyoxal, diethylpyrocarbonate, *N*-acetylimidazole do not produce a significative inhibition (data not shown). From the above results and taking into account that sulfhydryl groups are involved in the function of many transport proteins [29], protein SH groups were the target of our investigation.

Fig. 1 describes the effect of increasing concentrations of sulfhydryl reagents on the glycine transport activity in native membranes (A) and proteoliposomes (B). MalNEt, a membrane permeant reagent that alkylates SH groups, partially inactivated the glycine transport activity in membrane vesicles (75% max. inhibition), whereas only a slight inhibition (35%) was observed in proteoliposomes. MIANS, a more hydrophobic maleimide, inhibited the activity in a lesser extent (30%) than MalNEt in membrane vesicles and similar inhibition was observed with either maleimide in proteoliposomes (data not shown). In contrast, the hydrophilic organomercurials mersalyl and pCMBS displayed the greatest inhibitory effect on the transport activity regardless of the preparation used (95–100%). Inhibitory curves for these reagents were sigmoidal suggesting a complex chemical modification.

Organic mercurials are more specific for sulfhydryl groups than maleimide derivatives [25] and react with them forming mercaptides [26]. On the other hand, the hydrophilic nature of mersalyl and pCMBS do not allow these compounds to diffuse through the membrane readily [27]. Taken together, results shown on Table I and Fig. 1 indicate that there are certain cysteine residues on the glycine transporter involved in

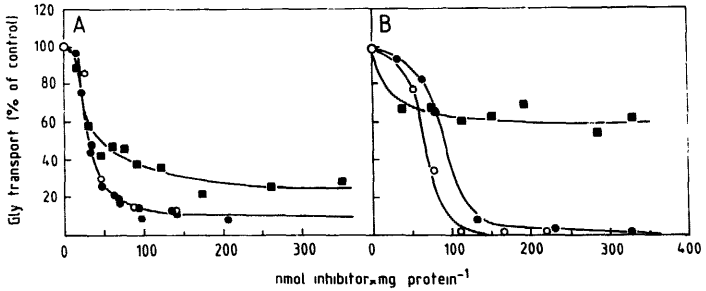

Fig. 1. Effects of different sulfhydryl groups reagents on glycine transport in membrane vesicles (A) or proteoliposomes (B). Synaptosomal membrane vesicles and proteoliposomes were pretreated with the indicated concentrations of chemical reagents for 10 min as indicated in Materials and Methods. After removing the unreacted reagents, glycine uptake was initiated. [^{3}H]Glycine transport in untreated membranes (27.5 pmol/mg protein per 2 min) (A) and untreated proteoliposomes (55 pmol/mg protein per 15 min) (B) was taken as 100% transport in each experiment. The following protein-modifying reagents were tested: *N*-ethylmaleimide (■), mersalyl (○), *p*-chloromercuribenzenesulfonate (●). Each point represents the mean of triplicate determinations in a representative experiment.

the activity of the protein as occurs for many other transport proteins [29–34], and probably located on the extracellular surface in a relatively hydrophilic environment. In this sense it is worthy to note that differences in the membrane environment as a result of alterations in lipid composition, lipid/protein ratio or interaction with extrinsic proteins are factors that contribute to differential reactivity with sulfhydryl reagents [32,35] probably through changes in the conformation of the carrier protein [38]. Membrane vesicles have a lipid/protein ratio of approx. 1:1, whereas the solubilized glycine transporter is reconstituted in liposomes at a lipid/protein ratio of 35:1 that imply a different membrane environment. Also, differences in the orientation of carrier proteins within the membrane (membrane vesicles versus proteoliposomes) may result in a different accessibility of some groups of the protein which could explain the different reactivity observed. The fact that the inhibitory effect shown in the presence of pCMBS and mersalyl can be reversed by excess DTT (Fig. 2) serves as further evidence for the involvement of sulfhydryl groups in the glycine transport process. The regeneration of activity indicates that the activity lost upon mercaptide formation is not due to an irreversible denaturation and that pCMBS and mersalyl have not reacted with groups other than sulfhydryl in the protein [26]. As expected, inactivation induced by irreversible SH reagents, such as MalNEt, cannot be reversed by DTT.

In the experiment shown in Fig. 3A, the time- and concentration-dependence of the effect of pCMBS on glycine transport activity was studied in more detail. A semilog plot of the fraction of remaining activity versus time is linear at each reagent concentration, suggesting that the inactivation is a pseudo-first-order process and likely due to a simple chemical event. A double-logarithmic plot of the pseudo-first-order inactivation rate constants, K_{obs}, versus pCMBS concentration [37] yields a minimal inhibition stoichiometry of 2.2 (Fig. 3A,

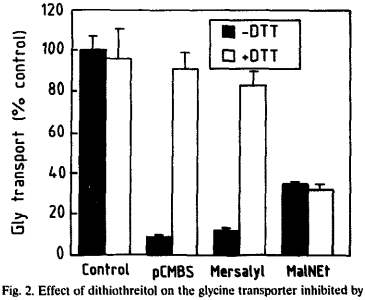

Fig. 2. Effect of dithiothreitol on the glycine transporter inhibited by sulfhydryl group reagents. Membrane vesicles were suspended in buffer A (Materials and Methods) and incubated in the presence of pCMBS and mersalyl (93 nmol/mg of protein), MalNEt (185 nmol/mg of protein) or none reagent (controls). After the reaction was finished and the unreacted reagent was removed as indicated, membrane vesicles were resuspended in the buffer A containing 5 mM DTT. After 10 min at room temperature excess DTT was removed and transport activity determinated. 100% corresponds to 29 pmol glycine/mg protein per 2 min. Data are values of three separate experiments, conducted on different membrane preparations, with three determinations (mean ± S.E.).

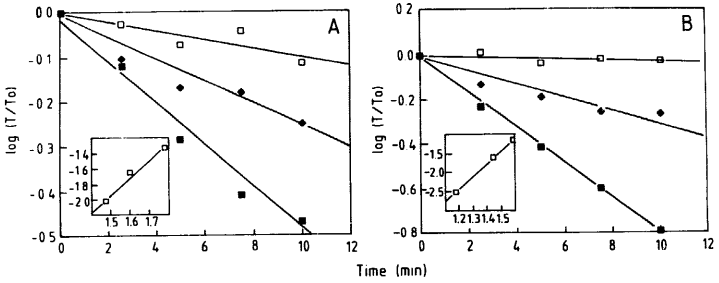

Fig. 3. Inactivation of the glycine transporter by pCMBS (A) and MalNEt (B). (A) Membrane vesicles were treated with 30 (□), 40 (◆), and 60 (■) nmol pCMBS/mg of protein, or (B) with 15 (□), 28 (◆), and 39 (■) nmol MalNEt/mg of protein as described in Materials and Methods. Samples were removed as a function of time and assayed for activity. Residual activities (T/T_0) at different times were plotted. The insets to panels A and B show the double-logaritmic plot of K_{obs} (observed inactivation rate constants) in ordinate,versus pCMBS and MalNEt concentrations (nmol/mg protein), respectively. Each point represents the mean of triplicate determinations in a representative experiment.

inset). These results suggest that two molecules of reagent are involved in the inactivation of the synaptosomal membrane vesicles glycine transporter, probably through the modification of two cysteine residues [28].

A similar experiment was performed using MalNEt (Fig. 3B). This reagent inhibits glycine transport in a concentration and time-dependent way; the plot of K_{obs} versus reagent concentration is also linear (Fig. 3B inset) and displays a slope of 3.3, suggesting that three cysteine residues are modified to promote inhibition of the glycine transport activity.

The nature of the effect of pCMBS and MalNEt on glycine transport activity was investigated by studying the initial-rate kinetics of glycine uptake in control conditions and in membranes pretreated with these reagents (Fig. 4). Kinetic analyses reveal that the diminished rate of transport observed in the presence of either reagent is the consequence of a decrease in the

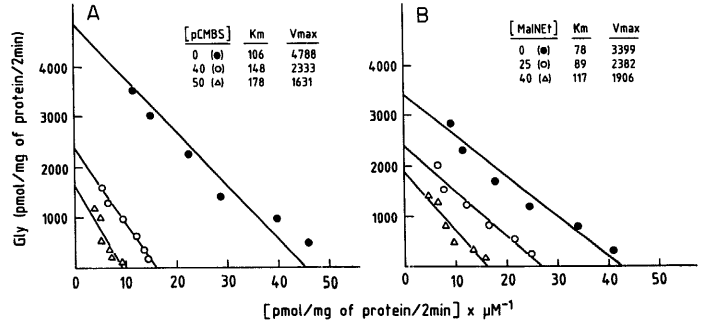

Fig. 4. Effect of pCMBS (A) and MalNEt (B) on the kinetics of glycine transport. Membrane vesicles were treated for 10 min as detailed in Materials and Methods. K_m and V_{max} values determinated for Eadie-Hofstee plots are summarized on the top of the figures. K_m values are indicated in μM and V_{max} as pmol Gly /mg of protein per 2 min. Linearization was obtained by least-squares, linear regression ($r > 0.9$ in all cases). Each point represents the mean of triplicate determinations in a representative experiment.

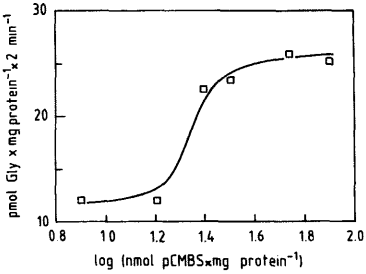

Fig. 5. Protective effect of pCMBS against irreversible inhibition by MalNEt. Membrane vesicles were treated 10 min with the indicated amount of pCMBS followed by addition of MalNEt (80 nmol/mg of protein) and after 10 min by DTT (10 mM). After each addition, excess reagent was removed as indicated in Materials and Methods. In the absence of any reagent, the activity corresponds to 31 pmol glycine/mg of protein per 2 min. Each point represents the mean of triplicate determinations in a representative experiment.

V_{max} with minor changes in the apparent K_m for glycine. pCMBS (50 nmol/mg protein) provoques a 2.9-fold and MalNEt (40 nmol/mg protein) a 1.8-fold decrease in the V_{max}. Additionally, a set of experiments were performed in order to know whether pCMBS and MalNEt react at the same or different thiol groups of the protein. Membrane vesicles were treated with the indicated amount of pCMBS (Fig. 5), followed by MalNEt (80 nmol /mg protein) and 10 mM DTT. Up to the concentration of 20 nmol/mg protein, pCMBS did not protect the glycine transport activity against the effect of the irreversible SH reagent MalNEt; however, the reactivation attained by DTT when pCMBS was present at 25 nmol/mg protein and above, indicates that pCMBS can protect the carrier against the action of MalNEt, suggesting that both reagents share some of the reactive thiol groups of the protein. On the other hand, the maximum inhibition displayed by MalNEt can be increased if the treatment with the maleimide is followed by the addition of a high concentration of pCMBS enough to produce the greatest inhibitory effect (Table II). These results indicate that after the treatment with MalNEt, some reactive SH groups remain unreacted on the protein, however, they are still able to interact with pCMBS. Taken together, results shown on Fig. 5 and Table II, suggest that both reagents could interact with the same and different SH groups on the transporter. Other possibility is that the blocking of SH groups by either reagent promotes a steric hindrance in the accessibility of the second reagent to different SH groups. Sulfhydryl groups which are involved in the transport function do not appear to participate in the formation of the substrate binding sites, since glycine, Cl^- and Na^+ ions fall to protect the activity against inhibition by pCMBS and MalNEt (data not shown). It could be that modification of thiol groups leads to loss of transport activity due to a steric effect and/or a conformational alteration with secondary effects. SH groups of proteins have been involved in conformational changes that affect the activity of different proteins [30,31,36]. Robillard and Konings [36] have suggested that the transport cycle of a number of cotransport systems involves a series of dithio–disulfide interchanges. Thus the reaction of these sulfhydryls with reagents would result in the inhibition of transport. Further experiments are required to establish whether SH groups here are involved in dithio–disulfide exchange.

TABLE II

Additive effect of pCMBS and MalNEt on glycine transport

Membrane vesicles were treated with the indicated reagents at a concentration of 150 nmol/mg of protein. After each addition, excess reagent was removed as indicated in Material and Methods. When MalNEt and pCMBS were used, membranes were treated in a sequential manner, MalNEt being used in the first place. Values are the means ± S.E. of three replicate experiments.

Treatment	Transport of glycine (pmol/mg protein per 2 min)
None	45.1 ± 2.2
pCMBS	0.7 ± 0.01
MalNEt	10.6 ± 0.3
MalNEt + pCMBS	0.6 ± 0.02

Conclusion

The results reported here demonstrate that sulfhydryl groups are involved in the function of the glycine transporter from rat brain stem and spinal cord. The localization of the essential sulfhydryl groups respect to the bilayer seems to be mainly at the outer, water-accessible surface of the protein either in membrane vesicles and proteoliposomes; but other SH groups participating with the glycine transporter activity in membrane vesicles may be located within hydrophobic domains of the tranporter which may include a protein-lipid complex. The exact role of the SH groups in transport is unknown; they do not participate in the formation of the substrate binding site of the carrier protein, but they probably have a role in the conformation changes necessary for the translocation step.

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