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Glycine taken up through GLYT1 and GLYT2 heterotransporters into glutamatergic axon terminals of mouse spinal cord elicits release of glutamate by homotransporter reversal and through anion channels

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Abstract

Glycine concentration-dependently elicited [^{3}H]D-aspartate ([^{3}H]D-ASP) release from superfused mouse spinal cord synaptosomes. Glycine effect was insensitive to strychnine or 5,7-dichlorokynurenic acid, but was prevented by the glycine transporter blocker glycyldodecylamide. Glycine also evoked release of endogenous glutamate, which was sensitive to glycyldodecylamide and abolished in low-Na^+ medium. Experiments with purified synaptosomes and gliasomes show that the glycine-evoked [^{3}H]D-ASP release largely originates from glutamatergic nerve terminals. The glycine-evoked [^{3}H]D-ASP release was halved by NFPS, a selective blocker of GLYT1 transporters, or by Org 25543, a selective GLYT2 blocker, and almost abolished by a mixture of the two, suggesting that activation of GLYT1 and GLYT2 present on glutamatergic terminals triggers the release of [^{3}H]D-ASP. Accordingly, confocal microscopy experiments show localization of GLYT1 and GLYT2 in purified synaptosomes immuno-stained for the vesicular glutamate transporter vGLUT1. The glycine effect was independent of extra- and intraterminal Ca^{2+} ions. It was partly inhibited by the glutamate transporter blocker DL-TBOA and largely prevented by the anion channel blockers niflumic acid and NPPB. To conclude, transporters for glycine (GLYT1 or/and GLYT2) and for glutamate coexist on the same spinal cord glutamatergic terminals. Activation of glycine heterotransporters elicits glutamate release partly by homotransporter reversal and largely through anion channels.

Keywords: Glutamate release; Glycine transporter types; Glycine heterotransporters; Glycine–glutamate interactions; Carrier-mediated release; Anion channels

1. Introduction

Glycine, GABA and glutamate are the major neurotransmitters in the mammalian spinal cord. Glycine plays important roles as an inhibitory transmitter acting at strychnine-sensitive receptors (for a review see [1]). Glycine and GABA coexist in subpopulations of spinal interneurons [2,3] and are thought to act as co-transmitters that can be co-released onto motoneurons [3,4]. Furthermore, glycine is the co-agonist of glutamate required for the activation of glutamate receptors of the NMDA type.

Interactions among glycine, GABA and glutamate can obviously be manifold and occur in various CNS regions, including the spinal cord. Many studies have investigated interactions at the postsynaptic level, whereas very little is known about the interactions that may occur in presynaptic nerve terminals of the spinal cord, if one excludes the modulations of transmitter release

Abbreviations: [^{3}H]D-ASP, [^{3}H]D-aspartate; BAPTA, 1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5,7-DCK, 5,7-dichlorokynurenic acid; GDA, glycyldodecylamide; GFAP, glial fibrillary acidic protein; NFPS, N[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; Org 25543, 4-benzyloxy-3,5-dimethoxy-*N*-[1-(dimethylaminocyclopentyl)methyl] benzamide; PSD-95, 95 kDa postsynaptic density protein; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; DL-TBOA, dl-threo-β-benzyloxyaspartic acid; vGLUT1, vesicular glutamate transporter type 1

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brought about by activation of presynaptic heteroreceptors [5].

In fact, other types of interaction can take place in CNS axon terminals, besides those mediated by heteroreceptors. There is evidence for the coexistence of neurotransmitter transporters on the same axon terminal, i.e. transporters, which recapture the transmitter just released (homotransporters) and transporters, which can take up different transmitters/modulators originating from neighbouring structures (heterotransporters). Activation of heterotransporters often provokes release of the transmitter synthesized in the terminals on which the heterotransporters are located (see, for reviews [6,7]). In the mouse spinal cord, we recently found that glycine can be taken up by selective heterotransporters into terminals endowed with GABA homotransporters and this uptake of glycine causes release of GABA in part by homotransporter reversal and in part by exocytosis [8].

The fact that glycine and glutamate are co-agonists at glutamate NMDA receptors prompted us to investigate the possible interactions between these neurotransmitters. The major aims of the present work were: (i) to ascertain if glutamate terminals in the spinal cord possess glycine heterotransporters using glycine transport inhibitors that have become available recently; (ii) to characterize pharmacologically the transporter types involved, using selective GLYT1 and GLYT2 blockers; (iii) to investigate the mechanisms of the glycine-evoked glutamate release. We here used synaptosomes prepared from the spinal cord of mice, also in view of the possible utilization of genetically modified animals.

2. Materials and methods

2.1. Animals

Adult female Swiss mice (weighing 20–25 g; Charles River, Calco, Italy) were used. Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (lights on 7 a.m.–7 p.m.). Food and water were freely available. All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, University of Genoa. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

2.2. Preparation of synaptosomes

Animals were sacrificed by cervical dislocation and the spinal cord was quickly removed. Crude synaptosomes were prepared essentially as described previously [9]. Briefly, the tissue was homogenized in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate, using a glass–Teflon tissue grinder (clearance 0.25 mm). In the experiments with BAPTA, the tissue was homogenized in the presence of 1 mM of the calcium chelator in order to entrap it into synaptosomes [10]. The homogenate was centrifuged (5 min, 1000 × *g* at 0–4 °C) to remove nuclei and debris, and synaptosomes were isolated from the supernatant by centrifugation at 12,000 × *g* for 20 min. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aeration with 95% O_2 and 5% CO_2); pH 7.2–7.4. Experiments were also performed with purified synaptosomes and gliasomes, which were prepared essentially according to Nakamura et al. [11]. Briefly, the supernatant of the 5-min centrifugation at 1000 × *g* was gently stratified on a discontinuous Percoll gradient (2, 6, 10 and 20%) and centrifuged at 33,500 × *g* for 5 min. The layers between 2 and 6% Percoll (gliasomal fraction) and between 10 and 20% (synaptosomal fraction) were collected, washed by centrifugation and resuspended in physiological medium.

2.3. Experiments of release

Synaptosomes (either crude or purified) or purified gliasomes were incubated at 37 °C for 15 min in the absence (experiments of endogenous glutamate release) or in the presence of 0.08 μM [^{3}H]D-ASP. Aliquots of the suspensions (about 50 or 9 μg protein in experiments with crude or purified synaptosomes, respectively; about 4 μg protein in experiments with gliasomes) were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37 °C [9]. Superfusion was then started with standard medium at a rate of 0.5 ml/min. After 33 min were allowed to equilibrate the system, five 3-min fractions were collected. Synaptosomes and gliasomes were exposed to glycine at the end of the second fraction collected (t = 39 min). Glycyldodecylamide (GDA), *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS), 4-benzyloxy-3,5-dimethoxy-*N*-[1-(dimethylaminocyclopentyl)methyl] benzamide (Org 25543), strychnine, 5,7-dichlorokynurenic acid (5,7-DCK), DL-threo-β-benzyloxyaspartic acid (DL-TBOA), niflumic acid or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was introduced at t = 30 min. Ca^{2+}-free medium (containing 10 mM Mg^{2+}) or Na^+-deficient medium was introduced at t = 20 min. When the Na^+-deficient medium was used, NaCl was replaced by an isoosmotic concentration of *N*-methyl-D-glucamine.

2.4. Neurotransmitter release determination

[^{3}H]D-ASP radioactivity was determined in each fraction collected and in the superfused filters by liquid

scintillation counting. Endogenous glutamate was measured by high performance liquid chromatography analysis following pre-column derivatization with *o*-phthalaldehyde and separation on a C_{18} reverse-phase chromatographic column (10 cm × 4.6 mm, 3 μm; at 30 °C; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm [10]). Homoserine was used as an internal standard.

2.5. Calculations

Tritium released in each fraction collected was calculated as fractional rate × 100. The endogenous glutamate release was expressed as pmol/mg of synaptosomal protein. Drug effects were evaluated by performing the ratio between the efflux in the fourth fraction collected (in which the maximum effect of glycine was generally reached) and that of the second fraction. This ratio was compared to the corresponding ratio obtained under control conditions. Appropriate controls were always run in parallel. The concentration–response curve shown in Fig. 1 was fitted to the experimental data using the following four parameters logistic equation, provided by the software Sigma Plot version 8.0: $y = a + \{(b - a)/[1 + (10^c/10^x)^d]\}$, where a is the minimum, b the maximum value, c the EC_{50} and d is the slope of the curve.

The two-tailed Student's *t*-test was used for statistical comparison of the data.

2.6. Immunoblotting

Aliquots of purified synaptosomes or gliasomes were analyzed to determine their glial fibrillary acidic protein (GFAP) and 95 kDa postsynaptic density protein (PSD-95) content. Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated at room temperature with the following concentrations of primary antibodies: 1:400 anti-GFAP monoclonal antibody, or 1:2000 anti-PSD-95 monoclonal antibody. After extensive washing and incubation with horseradish peroxidase-conjugated sheep anti-mouse secondary antibodies (1:2000 dilution), immunoreactivity was detected using the chemiluminescence system.

2.7. Immunofluorescence confocal microscopy

Purified synaptosomes (75 μg protein) were placed onto coverslips pre-treated with poly-l-ornithine and maintained 30 min at 37 °C in a 5% CO_2 atmosphere to allow setting and sticking to the substrate. All the following procedures were conducted at room temperature. The preparations were fixed with 2% paraformaldehyde (15 min), washed with PBS (3 × 5 min) and incubated (5 min) with 0.05% Triton X-100. After washing (3 × 5 min) with PBS containing 3% serum albumin, the preparations were incubated 30 min with the primary antibodies diluted in PBS containing 3% albumin. The following primary antibodies were used: goat anti-GLYT1, sheep anti-GLYT2, guinea pig anti-vesicular glutamate transporter type 1 (vGLUT1). After washing (3 × 5 min) with PBS containing 3% serum albumin, the preparations were incubated 30 min with the appropriate secondary fluorescein- or Alexa Fluor 594-labelled antibodies diluted in PBS containing 3% albumin and washed 3 × 5 min. Finally, synaptosomes were mounted on coverslips with FluoroGuard anti-fade reagent before analysis. Images were collected by confocal microscopy using a Bio-Rad MRC1024 instrument (krypton/argon laser, Bio-Rad, Hercules, CA, USA) attached to a Nikon Diaphot 200 inverted microscope (Nikon Inc., Melville, NY, USA), using a planapochromat X60 oil-

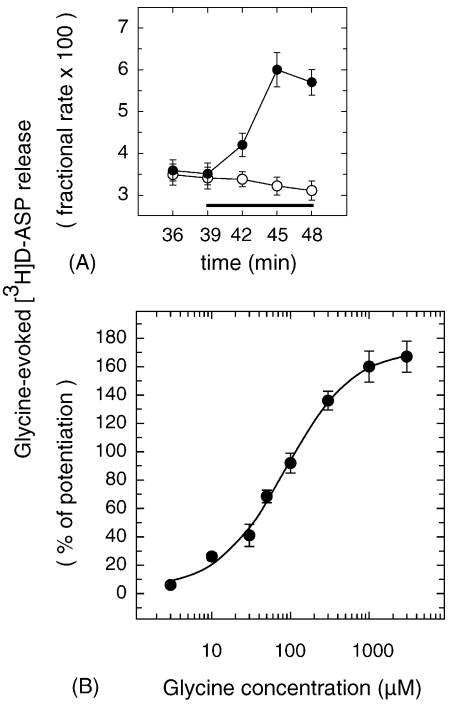

Fig. 1. Panel A: time-course of the release of [^{3}H]D-ASP elicited by glycine. Synaptosomes were labelled with the radioactive tracer and [^{3}H]D-ASP release monitored during superfusion. After 33 min were allowed to equilibrate the system, five 3-min fractions were collected. Synaptosomes were exposed to 100 μM glycine at the end of the second fraction collected (t = 39 min). Glycine was present until the end of the experiment (see line). Fractions were collected and counted for radioactivity. Results are expressed as fractional rate × 100. Data represent the mean ± S.E.M. of three experiments in triplicate (three superfusion chambers for each experimental condition). Solid circles: glycine-stimulated synaptosomes; empty circles: control synaptosomes. Panel B: concentration–response curve of the glycine-evoked [^{3}H]D-ASP release from mouse spinal cord synaptosomes. Synaptosomes were exposed in superfusion to various concentrations of glycine at the end of the second fraction collected. Results are expressed as per cent potentiation. Glycine effects were evaluated by performing the ratio between the efflux in the fourth fraction collected and that of the second fraction. This ratio was compared to the corresponding ratio obtained under control conditions. The data presented are mean ± S.E.M. of 3–10 experiments in triplicate.

immersion objective with N.A.1, 4. The excitation/emission wavelengths were 488/522 for fluorescein- and 567/605 for Alexa Fluor 594-labelled antibodies, respectively.

2.8. Drugs

[^{3}H]D-Aspartate (specific activity: 16.3 Ci/mmol) and horseradish peroxidase-conjugated sheep anti-mouse secondary antibody were purchased from Amersham (Buckinghamshire, UK). Glycine, strychnine, niflumic acid, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and the anti-GFAP monoclonal antibody (clone GA5) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); 5,7-dichlorokynurenic acid and DL-TBOA were from Tocris Cookson (Bristol, UK); 1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) from Fluka Biochemika (Milan, Italy); Percoll from Pharmacia (Uppsala, Sweden) and the anti-PSD-95 monoclonal antibody from Affinity Bioreagents Inc. (Golden, CO, USA). Anti-GLYT1 (AB1770), anti-GLYT2 (AB1771), anti-vGLUT1 (AB5905) primary antibodies and the donkey anti-guinea pig fluorescein-conjugated secondary antibody were obtained from Chemicon (Temecula, CA, USA). The donkey anti-sheep and the donkey anti-goat Alexa Fluor 594-conujugated secondary antibodies were purchased from Molecular Probes Europe (Leiden, The Netherlands). Glycyldodecylamide was donated by Abel Lajtha (Orangeburg, NY, USA); NFPS (ALX-5407) was a gift from NPS Allelix Corp (Mississauga, Ontario, Canada) and Janssen Research Foundation (Beerse, Belgium); Org 25543 was a gift from Dr. Hardy Sundaram (Organon Laboratories Ltd., Newhouse, Scotland).

3. Results

Fig. 1 shows that exogenous glycine, added to the superfusion medium, increased the spontaneous release of [^{3}H]D-ASP from mouse spinal cord crude synaptosomes pre-labelled with the radioactive amino acid. Panel A illustrates the time course of the potentiation evoked by 100 μM glycine: the spontaneous efflux of [^{3}H]D-ASP rapidly raised after exposure to glycine and reached a plateau during the fourth fraction collected. The releasing effect of glycine was concentration-dependent (Fig. 1, panel B): the maximal potentiation of release was about 170% over basal; the calculated EC_{50} value amounted to 87.2 μM.

The effect of 100 μM glycine on the release of [^{3}H]D-ASP was unaffected by 0.1 μM strychnine or 1 μM 5,7-DCK (Fig. 2), antagonists at the recognition site of the strychnine-sensitive glycine receptor and at the glycinergic co-agonist site of the NMDA glutamate receptor, respectively. Fig. 2 also shows that the release of [^{3}H]D-ASP elicited by 100 μM glycine was more than halved by 10 μM GDA, a compound reported to inhibit the uptake of [^{3}H]glycine into rat cortex synaptosomes [12]. Added at 50 μM, GDA almost completely abolished the glycine effect.

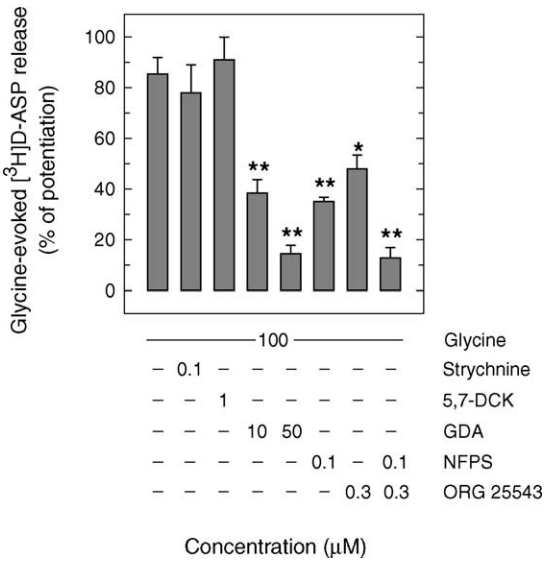

Fig. 2. Effects of the glycine receptor antagonist strychnine or 5,7-dichlorokynurenic acid (5,7-DCK) and of the glycine uptake inhibitors GDA, NFPS and Org 25543 on the release of [^{3}H]D-ASP induced by glycine from mouse spinal cord synaptosomes. Glycine was added to the superfusion medium at the end of the second fraction collected. Drugs were introduced 9 min before glycine. Results are expressed as percent potentiation of the basal efflux. Mean ± S.E.M. of three to five experiments in triplicate are reported. $^*P < 0.05$; $^{**}P < 0.01$ vs. the respective control value representing glycine in the absence of drugs (two-tailed Student's *t*-test).

Although [^{3}H]D-ASP, a non-metabolizable glutamate analogue, is frequently used in studies of glutamatergic transmission, its ability to perfectly mimic glutamate has sometimes been questioned. We therefore performed experiments in which the release of endogenous glutamate evoked by glycine from spinal cord synaptosomal preparations was monitored by HPLC. Table 1 shows that 100 μM glycine potentiated endogenous glutamate release to an extent (~75%) similar to that of [^{3}H]D-ASP release. The table also shows that the effect of 100 μM glycine was largely prevented by 50 μM GDA and almost abolished when the [Na^+] in the superfusion medium was lowered to 23 mM.

Another point that requires careful consideration, particularly in studies of glutamate uptake and release with synaptosomal preparations, is the possible contamination by gliasomes, since glia is remarkably rich of glutamate transporters and has been reported to release glutamate [13–15]. Furthermore, glial cells have been shown to possess glycine transporters [16–18]. It was therefore important to evaluate the possible contribution of gliasomes to the glycine-evoked glutamate release. To this aim both spinal cord synaptosomes and gliasomes were purified according to Nakamura et al. [11]. Fig. 3 illustrates the pattern of distribution of the neuronal marker PSD-95 (panel A) and of the glial marker GFAP (panel B) obtained

Table 1
Effects of glycyldodecylamide (GDA), Na^+ deprivation and of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on the glycine-evoked release of endogenous glutamate from mouse spinal cord synaptosomes

Drug	Percentage of potentiation (mean ± S.E.M.)	*n*
100 μM glycine (in standard medium, containing 145 mM Na^+)	73.8 ± 8.5	7
100 μM glycine (in standard medium) + 50 μM GDA	25.8 ± 2.0[a]	3
100 μM glycine (in 23 mM Na^+-containing medium)	7.9 ± 11.4[a]	3
100 μM glycine (in standard medium) + 100 μM NPPB	17.2 ± 2.5[b]	5

Glycine was present from the end of the second fraction collected throughout the experiment. The 23 mM Na^+-containing medium was introduced 19 min before glycine. GDA and NPPB were added 9 min before glycine. Results are expressed as percent potentiation of the spontaneous efflux of endogenous glutamate. The spontaneous efflux of glutamate in the second fraction collected (control basal release) amounted to: 309 ± 31 pmol/mg protein ($n = 7$). The data are mean ± S.E.M. of three to seven experiments in triplicate.

[a] $P < 0.05$ vs. the respective control value representing glycine in the absence of drugs (two-tailed Student's *t*-test).

[b] $P < 0.01$ vs. the respective control value representing glycine in the absence of drugs (two-tailed Student's *t*-test).

by Western blot analysis of the two preparations. The analysis shows some gliasomal GFAP contamination in the purified synaptosomal fraction, while the gliasome fraction appears very weakly contaminated by synaptosomes. Panel C of Fig. 3 shows that the release of pre-accumulated [^{3}H]D-ASP elicited by glycine was much more pronounced in purified synaptosomes (~120% over basal with 100 μM glycine) than in purified gliasomes (~25% over basal). Since the response of purified synaptosomes to glycine is much stronger than that of purified gliasomes, the gliasomal contamination seen in purified synaptosomes (Fig. 3B) is unlikely to provide important contribution to the glycine-evoked release from crude synaptosomes; on the other hand, the few synaptosomes present in the gliasome fraction (Fig. 3A) could account for the modest release of [^{3}H]D-ASP caused by glycine when added to gliasomes, although a small glycine-evoked release from gliasomes can not be entirely ruled out. In any case, it may be concluded that the potentiation of [^{3}H]D-ASP release provoked by glycine when crude synaptosomal preparations are used essentially occurs in glutamatergic nerve terminals with little contribution by gliasomes.

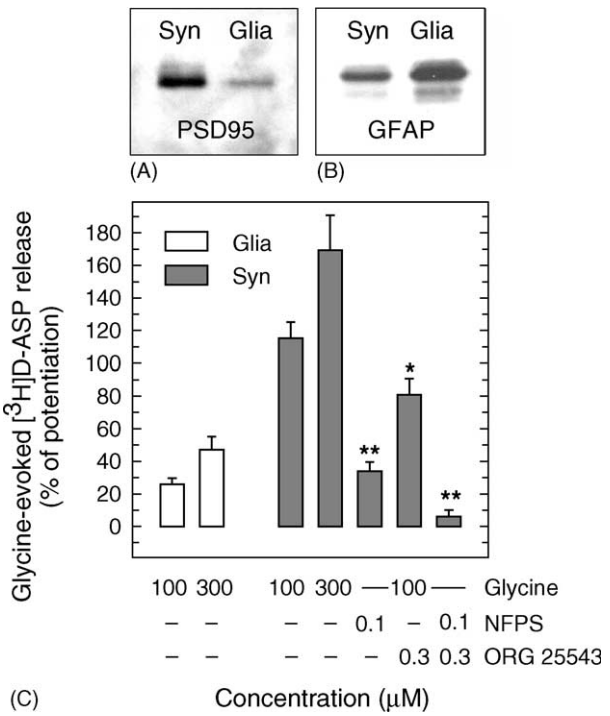

Fig. 3. Panels A and B: PSD-95 (A) and GFAP (B) blots from purified spinal cord synaptosomes (Syn) or gliasomes (Glia). Synaptosomes (10 μg per lane) and gliasomes (10 μg per lane) were subjected to SDS–PAGE followed by Western blotting using mouse anti-GFAP or anti-PSD-95 monoclonal antibodies. Detection was performed with an Amersham ECL system. Panel C: effects of glycine and the glycine transport blockers NFPS and Org 25543 on the release of [^{3}H]D-ASP from purified spinal cord gliasomes and synaptosomes. Purified gliasomes or synaptosomes were prepared using a discontinuous Percoll gradient and exposed in superfusion to glycine starting from the second fraction collected. NFPS or Org 25543 was introduced 9 min before glycine. The release of tritium in the second fraction collected (control basal release) amounted to 4.60 ± 0.39 ($n = 10$) and to 4.82 ± 0.33 ($n = 10$) of the total gliasomal and synaptosomal tritium content, respectively. Results are expressed as per cent potentiation of the basal release. The data presented are mean ± S.E.M. of four to five experiments in triplicate. $^*P < 0.05$ and $^{**}P < 0.01$ vs. the respective glycine-induced potentiation measured in synaptosomes (two-tailed Student's *t*-test).

The results with GDA suggest the involvement of glycine transporters in the glycine-evoked [^{3}H]D-ASP release. Two types of glycine transporter have been identified and termed GLYT1 and GLYT2 ([17–19] see, for a review [20]). GLYT1 and GLYT2 can be pharmacologically distinguished by some non-transportable selective blockers. One of these transporter inhibitors is the potent and selective GLYT1 blocker NFPS [21,22]. Added at 0.1 μM, NFPS prevented by almost 60% the glycine (100 μM)-evoked [^{3}H]D-ASP release (Fig. 2). Slightly lower inhibition was produced by 0.3 μM of Org 25543, a selective GLYT2 blocker [23]. These figures represent the maximal effects observed: 1 μM of NFPS or Org 25543 did not produce any further inhibition of the glycine-evoked [^{3}H]D-ASP release (data not shown). The figure also shows that, when the two transporter blockers were added together, their effects were additive. The releasing effect of glycine (100 μM) also could be inhibited by NFPS (0.1 μM) or Org 25543 (0.3 μM) in purified synaptosomes (Fig. 3C), with NFPS appearing significantly more potent than Org 25543. Quantitatively similar effects could be observed by adding 1 μM of the transporter blockers (not shown).

The results obtained from confocal microscopy experiments are illustrated in Fig. 4. Purified synaptosomes were

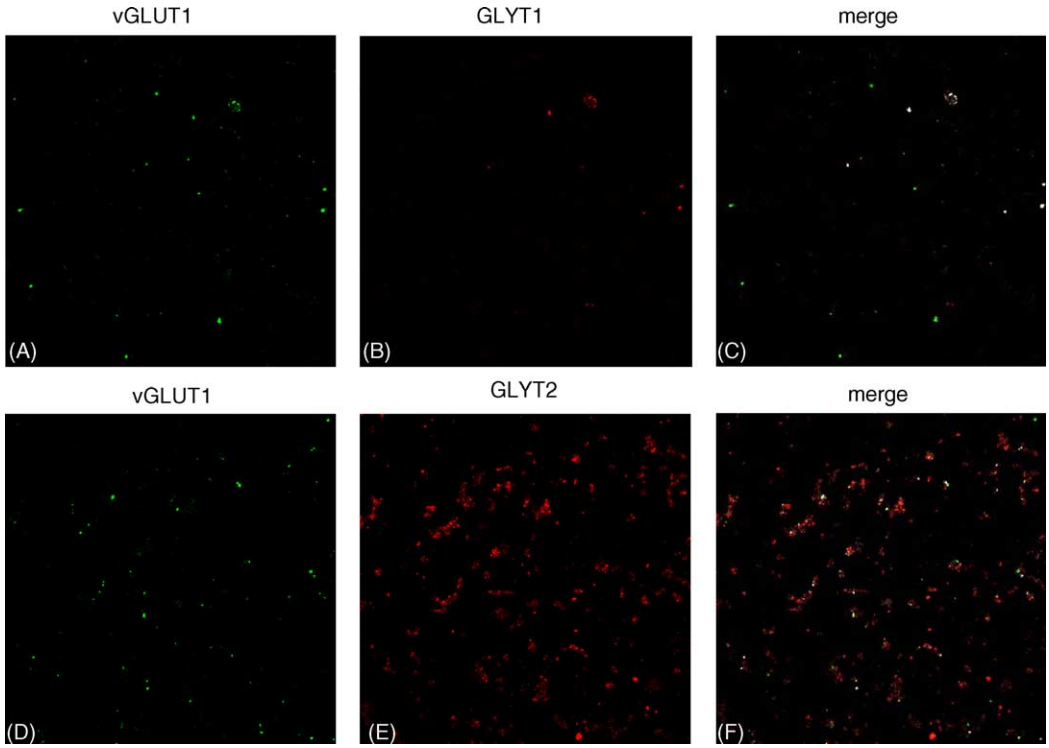

Fig. 4. Identification of the vesicular type 1 glutamate transporter (vGLUT1) and membrane type 1 (GLYT1) and type 2 (GLYT2) glycine transporters in purified spinal cord synaptosomes by immunocytochemistry. Synaptosomes were glued onto coverslips, fixed with paraformaldehyde, permeabilized with Triton X-100 and incubated with the primary and secondary antibodies. Images show the fluorescein-tagged anti-vGLUT1 (A, D) and the Alexa Fluor 594-tagged anti-GLYT1 (B) or anti-GLYT2 (E). The yellow color represents co-expression of vGLUT1 and GLYT1 (C) or vGLUT1 and GLYT2 (F).

labelled with anti-vGLUT1 (green, panels A and D) and with anti-GLYT1 (red, panel B) or anti-GLYT2 (red, panel E) antibodies. The synaptosomal preparation efficiently stained for vGLUT1 and for GLYT2. Labelling for the reported preferential glial transporter GLYT1 was less noticeable (panel B). The majority of the visible GLYT1 positive particles co-localize with vGLUT1-positive particles, while few of the vGLUT1-positive synaptosomes was labelled by the GLYT1 antibody (Fig. 4C). As to the other glycine transporter, a number of the very numerous purified synaptosomes showing GLYT2 expression also expressed vGLUT1 (Fig. 4F). These experiments suggest that GLYT1 and GLYT2 transporters could be expressed on glutamatergic nerve endings in mouse spinal cord, although at least part of the particles exhibiting both vGLUT1 and GLYT1 staining may represent gliasomal contamination.

Experiments were then performed to shed light on the mechanism(s) by which [^{3}H]D-ASP exits from mouse spinal cord nerve terminals when these are exposed to glycine. Superfusion of synaptosomes with Ca^{2+}-free medium did not modify significantly the effect of glycine (Fig. 5). Since vesicular exocytosis also can be triggered by Ca^{2+} ions entering the cytosol from intraterminal stores, experiments were carried out with synaptosomes in which the Ca^{2+} chelator BAPTA had been entrapped by homogenizing the spinal cord in the presence of 1 mM of the membrane-impermeant Ca^{2+} chelator. Using this technique [10,24], the intraterminal concentration of the entrapped compound should be about 5% of the original concentration present in the homogenization medium (in the case of BAPTA, ≈50 μM). Entrapping BAPTA should avoid possible undesired effects on K^+ channels reported to occur with the membrane-permeant BAPTA-AM [25]. Fig. 5 shows that the entrapped Ca^{2+} chelator did not modify the effect of glycine on the release of [^{3}H]D-ASP. Under identical experimental conditions, entrapped BAPTA had been found to reduce by ≈50% the release of [^{3}H]GABA elicited by glycine from mouse spinal cord synaptosomes [8] as well as the release of glutamate evoked by high KCl [10]. The glycine (100 μM)-evoked [^{3}H]D-ASP release was prevented by about 30% in the presence of 100 μM of the glutamate transporter blocker DL-TBOA (Fig. 5). Fig. 5 also shows that the effect of glycine on the release of [^{3}H]D-ASP was strongly reduced by two anion channel blockers, NPPB (10 and 100 μM) or niflumic acid (300 μM). As shown in Table 1, NPPB (100 μM) also strongly inhibited the glycine (100 μM)-evoked release of endogenous glutamate.

4. Discussion

4.1. Methodological aspects

Synaptosomal preparations, either crude or purified, contain several different families of nerve endings. To

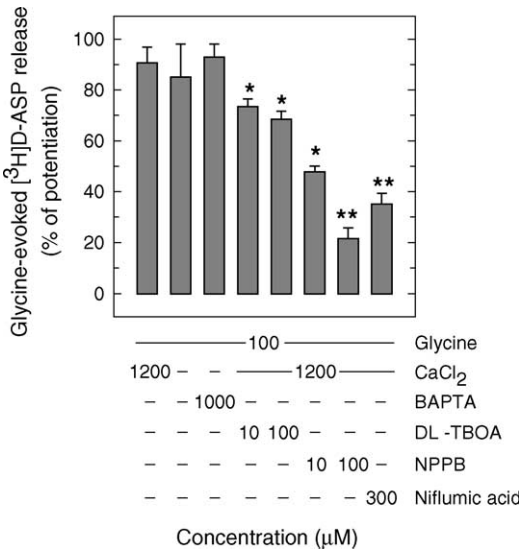

Fig. 5. Effects of Ca^{2+} omission, of the Ca^{2+} chelator BAPTA, of the non-transportable glutamate carrier blocker DL-TBOA, and of the anion channel blockers niflumic acid and NPPB on the release of [^{3}H]D-aspartate induced by glycine from spinal cord synaptosomes. Glycine was present from the end of the second fraction collected. BAPTA was entrapped into synaptosomes during the homogenization of the tissue. Calcium was omitted during superfusion, 19 min before the introduction of glycine. DL-TBOA, niflumic acid or NPPB was introduced 9 min before glycine. Results are expressed as percent potentiation of the basal release. The data are mean ± S.E.M. of three to five experiments performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the respective control value representing glycine in the absence of drugs (two-tailed Student's *t*-test).

ascertain whether glycine affects directly the release of glutamate, possible indirect effects mediated by other compounds have to be minimized. To this aim, synaptosomal suspensions were layered on microporous filters (diameter 2.5 cm) in such an amount (≪100 μg protein/filter) that, based on previous estimates [26], the particles constitute less than a monolayer. Many studies have demonstrated that, when such a synaptosomal layer is up–down superfused, any compound released is rapidly removed before it can activate the targets present in the preparation (transporters, presynaptic receptors and so on). Therefore all these targets remain virtually ligand-free, but can be selectively activated by appropriate ligands added to the superfusion medium (see, for details [9]). Under such conditions, the changes of glutamate release produced by compounds (glycine, in the present work) added to the superfusion medium are due to their direct action on glutamate-releasing particles. The use of this technique permits to functionally establish the co-localization, on a given family of nerve endings, of two different receptors, or two different transporters (present work) and to investigate their cross-talks.

Studies of glutamate/aspartate release are often performed by pre-labelling brain tissues with [^{3}H]D-ASP, assuming that the compound perfectly mimics the endogenous excitatory amino acid transmitters. The advantages of using [^{3}H]D-ASP are quite relevant, considering that the amino acid is taken up by glutamate transporters and it is non-metabolizable. On the other hand, that [^{3}H]D-ASP compartmentalization and release exactly parallel those of the endogenous counterpart has at times been questioned. For this reason, a group of experiments was performed in which the release of endogenous glutamate from spinal cord synaptosomes was monitored. Based on the results reported in Table 1, the behaviour of endogenous glutamate appears indistinguishable from that of [^{3}H]D-ASP, which justifies the use of the tritiated analogue in these kind of studies.

4.2. Glycine targets on glutamate-releasing terminals are glycine transporters of both the GLYT1 and GLYT2 types

The elevation of glutamate release observed when synaptosomal preparations are exposed to exogenous glycine in superfusion suggests that certain glutamate-releasing axon terminals in the mouse spinal cord possess targets for glycine. The effect of glycine was insensitive to 5,7-DCK, a selective antagonist at the glycine site of the NMDA receptor, which excludes that glycine acts at release-stimulating NMDA receptors. Glycine has been reported to induce neurotransmitter release directly via strychnine-sensitive receptors, probably exploiting an in-out direction of the chloride gradient (see, for instance [27]). However, in our experiments, the glycine effect was not sensitive to strychnine, thus excluding the involvement of the ionotropic glycine receptor.

The Na^+-dependence of the glycine-evoked glutamate release and its sensitivity to the glycine transporter inhibitors glycyldodecylamide, NFPS and Org 25543 indicate that glycine uptake is the primary trigger for glutamate release. Thus, also considering the characteristics of the superfusion technique described above, axon terminals able to take up [^{3}H]D-ASP, and which therefore possess glutamate transporters, seem to co-express transporters for glycine, the activation of which elevates the basal release of glutamate. In our synaptosomal preparation glycine evoked glutamate release with an $EC_{50} = 87.2$ μM, comparable with reported K_m values for glycine uptake by transiently or stably expressed GLYT1 and GLYT2 transporters [28].

As previously mentioned, glycine transporters are heterogeneous: two types, termed GLYT1 and GLYT2, have been identified and molecularly characterized (for a recent review see [20]), but their function is only in part understood. GLYT2 is thought to be a neuronal transporter, specifically associated with inhibitory glycinergic neurotransmission, because it is localized on glycine-immunoreactive neurones, at the presynaptic side of glycinergic synapses in the spinal cord [17,18,29]. As to GLYT1, this transporter was shown to be expressed in glia [16,17], although its presence in neurones also has sometimes been proposed [8,17].

Our functional results clearly indicate that crude synaptosomal preparations from mouse spinal cord contain both

GLYT1 and GLYT2 (see Fig. 2). In particular, the glycine-evoked release of glutamate consists of two components, one sensitive to the GLYT1 selective inhibitor NFPS and the other sensitive to the GLYT2 selective inhibitor Org 25543. As expected, the NFPS- and Org 25543-sensitive components are additive.

4.3. Glycine-evoked glutamate release from purified gliasomes and synaptosomes

Crude synaptosomal preparations are known to contain contaminating gliasomes, fragments of glial cells produced during tissue homogenization and then resealed. The viability of these particles has been in part controversial (see [30] for discussion). A gliasome fraction was isolated from adult rat brain and found able to accumulate [^{3}H]GABA and [^{3}H]D-ASP [11]. In order to evaluate the contribution of gliasomes to the glycine-evoked [^{3}H]D-ASP release from our crude synaptosomal preparations, purified gliasomes and synaptosomes were prepared according to Nakamura et al. [11]. The Western blot analysis of the fractions shows that the gliasome fraction contains very few contaminant synaptosomes while the synaptosomal fraction still contains some contaminant gliasomes. On the other hand, the increase of [^{3}H]D-ASP release provoked by glycine in gliasomes was only 30% over basal whereas that from synaptosomes amounted to ~120%. It may be reasonable to conclude that, if transporters of the GLYT1 type exist on gliasomes, they are poorly coupled to glutamate release; on the other hand, transporters of the GLYT1 type well coupled to glutamate release seem to be present in purified nerve terminals. Gliasomes appear to contribute modestly to the effect of glycine observed in crude synaptosomal preparation. It cannot be excluded, however, that glutamate can be efficiently released following GLYT1-mediated glycine uptake into intact adult glial cells.

4.4. Localization of glycine heterotransporters on glutamatergic nerve terminals by confocal microscopy

The functional results obtained by monitoring the release of glutamate elicited by glycine from superfused synaptosomes strongly suggest that glutamate homotransporters and glycine heterotransporters coexist on mouse spinal cord nerve endings. We attempted to obtain additional evidence for the presence of glycine transporters on glutamate-storing terminals by performing experiments of confocal microscopy with purified synaptosomal preparations. Assuming that vGLUTs are selective markers for axon terminals that store and release glutamate, the localization of anti-vGLUT1 antibodies together with anti-GLYT1 or anti-GLYT2 antibodies would indicate that glycine heterotransporters are present on glutamatergic nerve endings in the spinal cord. Transporters of the GLYT1 type were reported to be preferentially localized on glial cells [16,17]. The relatively poor staining for GLYT1 in purified synaptosomes (Fig. 4B) is in keeping with the low contamination by gliasomes. On the other hand, the majority of GLYT1 expressed in purified synaptosomes co-localized with vGLUT1, compatible with their presence on glutamatergic terminals where, based on the results of release experiments, they mediate a prominent portion of the glycine-evoked glutamate release (Fig. 3). Differently from GLYT1, GLYT2 exhibits a pronounced staining. Because glycinergic neurons in the spinal cord are endowed with transporters of the GLYT2 type [29], most of the particles that stain for GLYT2 are likely to be glycinergic nerve endings carrying GLYT2 homotransporters. These terminals should not contain vesicular glutamate transporters, as recently demonstrated by Todd et al. [31]. At least part of the remaining GLYT2-bearing particles consists of glutamatergic terminals, which stain for vGLUT1. Thus, although the pictures obtained by confocal microscopy do not allow quantitative evaluation, they seem to strengthen the conclusion drawn from the functional data.

If the results obtained in this work seem to indicate that glycine heterotransporters are located on a number of glutamatergic axon terminals in the spinal cord, it is at present unknown if a given glutamatergic terminal possesses both GLYT1 and GLYT2 or if there exist glutamatergic terminals on which glutamate transporters coexist with either GLYT1 or GLYT2. The latter possibility seems at present more likely, considering that GLYT1 and GLYT2 display a completely different expression pattern in the spinal cord [16–18]. All together the results available can allow to conclude that: (i) transporters of the GLYT1 type are present not only on glia [16,17], but also on glutamatergic axon terminals of the mouse spinal cord, as heterotransporters; (ii) GLYT2 are present not only on the terminals of inhibitory glycinergic neurons as reuptake homotransporters [17,18,29], but also, as heterotransporters, on glutamatergic (present work) and GABAergic [8] axon terminals in the mouse spinal cord; (iii) GLYT1 and GLYT2 heterotransporters sited on glutamatergic terminals can capture extracellular glycine originating from yet unidentified structures including glycinergic terminals and astrocytes [32]; (iv) although the primary function of GLYT1 and GLYT2 may be (re)uptake into glia and glycinergic neurons, respectively, the present results and the previously published data on the glycine–GABA interaction [8] suggest a possible additional function for the spinal glycine transporters, i.e. to mediate regulation of the basal release of the two major inhibitory and excitatory transmitters GABA and glutamate.

It has been reported that the distribution of GLYT1 correlates better with the localization of NMDA receptors than with the strychnine-sensitive glycine receptor [18]. One could therefore speculate that glycine activates GLYT1 on glutamatergic terminals to facilitate glutamate release onto NMDA receptors; the opposite might be unnecessary because relatively high concentrations of glycine are already present in the extracellular space [33].

4.5. Mode of exit of glutamate during the glycinergic stimulus

Vesicular exocytosis is the physiological mechanism by which axon terminals release neurotransmitters. Classical exocytosis triggered by Ca^{2+} entering through voltage-sensitive Ca^{2+} channels seems however not to be involved in the efflux of glutamate evoked by glycine. In fact, the effect of glycine is independent of external Ca^{2+} and therefore occurs in the absence of voltage-sensitive Ca^{2+} channel activation. Exocytosis can also occur when Ca^{2+} ions reach the cytosol following mobilization from intraterminal stores. However, the effect of glycine was not significantly reduced in synaptosomes containing the Ca^{2+} chelator BAPTA. Thus, the glycinergic potentiation of glutamate release appears independent of external and internal Ca^{2+}, excluding that glycine transporter activation can enhance glutamate release by stimulating vesicular exocytosis.

In some conditions, neurotransmitters can be released directly from the cytosol through the homotransporter working in the inside-out direction (see [34,35], for reviews). This carrier-mediated release is Ca^{2+}-independent and sensitive to homotransporter inhibitors [36]. As can be seen in Fig. 4, DL-TBOA, a non-transported blocker of glutamate carriers [37], inhibited by about 30% the glycine-evoked release, indicating that a portion of glutamate exits by homotransporter reversal, possibly facilitated by Na^+ ions co-transported with glycine by the plasma-membrane glycine transporters [20]. If only a fraction of the glutamate released by glycine exits through a carrier-mediated process and exocytosis has to be excluded, how could the glycine-evoked release of glutamate occur?

Cell membranes possess families of various Cl^-/anion channels. Among these, the volume-sensitive outwardly rectifying anion channels (VSOAC) are ubiquitously expressed in mammalian cells where they participate in the regulatory volume decrease mechanism. VSOAC are not only permeable to Cl^-, but also to organic osmolytes. Their pharmacology includes sensitivity to niflumic acid and NPPB [38,39]. Anion channel-mediated release of amino acids, including glutamate, has been observed in various brain preparations including glial cells and nerve endings [40,41]. Activation of VSOAC generally occurs during swelling caused by hypotonic solutions. However, evidence exists that some anion channels not only open in response to large decreases of external osmolarity, but also under near isotonic conditions. It was recently shown that [^{3}H]D-ASP can be released through anion channels in hippocampal slices after small and gradual increases in intracellular osmolarity [42]. In this context, the entry of Cl^- ions co-transported with glycine might be sufficient to trigger Cl^-/anion channel activation. Interestingly, glutamate was shown to be released through anion channels from cerebellar parallel fiber terminals/varicosities during activation of $GABA_A$ receptors [41]. Of note, GABA, which is not in the anion form at physiological pH, when released during glycine heterotransporter (GLYT1 and GLYT2) activation in mouse spinal cord synaptosomes, does not exit through anion channels but by exocytosis and homotransporter reversal [8], indicating that activation of the same glycine heterotransporters sited on different neuron terminals does not elicit transmitter release by identical mechanisms.

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