Activation of γ-Aminobutyric Acid GAT-1 Transporters on Glutamatergic Terminals of Mouse Spinal Cord Mediates Glutamate Release Through Anion Channels and by Transporter Reversal

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The effects of γ-aminobutyric acid (GABA) on the release of glutamate from mouse spinal cord nerve endings have been studied using superfused synaptosomes. GABA elicited a concentration-dependent release of [3H]D-aspartate ([3H]D-ASP; EC50 = 3.76 μM). Neither muscimol nor (–)baclofen mimicked GABA, excluding receptor involvement. The GABA-evoked release was strictly Na+ dependent and was prevented by the GABA transporter inhibitor SKF89976A, suggesting involvement of GAT-1 transporters located on glutamatergic nerve terminals. GABA also potentiated the spontaneous release of endogenous glutamate; an effect sensitive to SKF89976A and low-Na+-containing medium. Confocal microscopy shows that the GABA transporter GAT-1 is coexpressed with the vesicular glutamate transporter vGLUT-1 and with the plasma membrane glutamate transporter EAAT2 in a substantial portion of synaptosomal particles. The GABA effect was external Ca2+ independent and was not decreased when cytosolic Ca2+ ions were chelated by BAPTA. The glutamate transporter blocker DL-TBOA or dihydrokainate inhibited in part (~35%) the GABA (10 μM)-evoked [3H]D-ASP release; this release was strongly reduced by the anion channel blockers niflumic acid and NPPB. GABA, up to 30 μM, was unable to augment significantly the basal release of [3H]glycine from spinal cord synaptosomes, indicating selectivity for glutamatergic transmission. It is concluded that GABA GAT-1 transporters and glutamate transporters coexist on the same spinal cord glutamatergic terminals. Activation of these GABA transporters elicits release of glutamate partially by reversal of glutamate transporters present on glutamatergic terminals and largely through anion channels.

Key words: GABA transporters; glutamate release; glycine release; transporter coexistence; spinal cord synaptosomes

Transporters mediating reuptake of neurotransmitters have long been considered as selective neuronal markers. However, there is increasing evidence that neurons can coexpress reuptake transporters together with transporters able to capture various other transmitters/modulators. Important examples are the glutamate transporters of the EAAT4 type, which are localized on γ-aminobutyric acid (GABA)-ergic Purkinje cells in the cerebellum (Rothstein et al., 1994; for review see Furuta et al., 1997), the transporters of the EAAT3 type present on hippocampal GABAergic neurons (Sepukti et al., 1997; Raiteri et al., 2002) and the glutamatergic transporters found on cholinergic spinal motoneurons (Shashidharan et al., 1997). Heterologous transporters also have been found to exist on the terminals of several neurons and to exhibit cell selectivity and regional heterogeneity (for review see Bonanno and Raiteri, 1994; Raiteri et al., 2002). However, although coexistence of transporters on the same neuron seems to be a widespread phenomenon, its function and the mechanisms involved in transporter interaction are far from being understood.

Glutamate, GABA, and glycine are the major neurotransmitters in the mammalian spinal cord. Several studies have investigated interactions among these transmitters at the postsynaptic level, whereas relatively little is known about the interactions that may occur in presynaptic terminals. One of the mechanisms suggested for this interaction is anion channel-mediated release of glutamate (Haas et al., 1987; Haas, 1990). This release is strongly opposed by the presence of anion channel blockers like niflumic acid and NPPB (Haas et al., 1987). The GABA transporter-mediated release of glutamate is also strongly opposed by niflumic acid (Raiteri et al., 1995). This suggests that the GABA transporters are interacting with the anion channel to cause glutamate release.

In conclusion, the GABA transporters on mouse spinal cord nerve endings appear to mediate glutamate release through anion channel and transporter reversal mechanisms. The activation of these transporters is selective for glutamatergic transmission, as demonstrated by the inability of GABA to augment significantly the basal release of [3H]glycine from spinal cord synaptosomes. The coexistence of GABA and glutamate transporters on the same neuron provides a mechanism for the modulation of neurotransmitter release and suggests a novel role for GABA transporters in regulating glutamate transmission. Further studies are needed to clarify the cellular and molecular mechanisms underlying these interactions.

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naptic nerve terminals of the spinal cord. We recently found that glycine can be taken up by selective transporters into nerve terminals endowed with GABA transporters and that the uptake of glycine elicits release of GABA in part by transporter reversal and in part by exocytosis (Raïteri et al., 2001a). In this case, it should be noted that glycine and GABA coexist in subpopulations of spinal interneurons (Todd et al., 1996) and are thought to act as cotransmitters that can be coreleased onto motoneurons (Todd et al., 1996; Jonas et al., 1998). Glycine and GABA, once coreleased, are expected to be recaptured into the releasing terminals by selective transporters, so coexpression of glycine and GABA transporters might reflect only cotransmission (Burnstock, 2004).

It was therefore important to investigate the existence and the properties of transporters sited on terminals not storing the corresponding transmitter. We chose to study the interactions between GABA and glutamate, which have not been reported to be cotransmitters in the mouse spinal cord. The present work was performed by monitoring \[^3H\]D-aspartate (\[^3H\]D-ASP) or endogenous glutamate release evoked by GABA from thin layers of synaptosomes in superfusion, a preparation well suited to establish colocalization of transporters/receptors on the same terminal and characterize their possible cross-talk (see Raïteri and Raïteri, 2000). Our findings suggest that GABA and glutamate transporters coexist on glutamatergic terminals in the spinal cord; activation of GABA transporters can enhance spontaneous glutamate release by two mechanisms: transporter reversal and activation of anion channels.

**MATERIALS AND METHODS**

**Animals**

Adult female Swiss mice (weighing 20–25 g; Charles River, Calco, Italy) were used. Animals were housed at constant temperature (22°C ± 1°C) and relative humidity (50%) under a regular light–dark schedule (lights on from 7 AM to 7 PM). 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 with the NIH Guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

**Preparation of Synaptosomes**

Animals were sacrificed by cervical dislocation, and the spinal cord was quickly removed. Synaptosomes were prepared essentially as described previously (Raïteri and Raïteri, 2000). Briefly, the tissue was homogenized in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate, by using a glass-teflon tissue grinder (clearance 0.25 mm). In the experiments with 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), the tissue was homogenized in the presence of 1 mM of the calcium chelator in order to entrap it into synaptosomes (Raïteri et al., 2000). The homogenate was centrifuged (5 min, 1,000 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\(_2\)PO\(_4\) 1, NaHCO\(_3\) 22, glucose, 10 (aeration with 95% \(\mathrm{O_2}\) and 5% \(\mathrm{CO_2}\)), pH 7.2–7.4.

**Experiments of Release**

Synaptosomes were incubated at 37°C for 15 min in the absence (experiments of endogenous glutamate release) or in the presence of 0.08 \(\mu\)M \[^3H\]D-ASP or of 0.3 \(\mu\)M \[^3H\]glycine. Aliquots of the suspensions (about 50 \(\mu\)g protein) were distributed on microporous filters placed at the bottom of a parallel superfusion chambers maintained at 37°C (Raïteri and Raïteri, 2000). 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 were exposed to GABA, muscimol, or (±)baclofen at the end of the second fraction collected (\(t = 39\) min). N-(4,4-diphenyl-3-butenyl)nipecotic acid (SKF 89976A), DL-threo-β-benzoxylaspartic acid (DL-TBOA), dihydrokainic acid (DHK), niflumic acid, or 5-nitro-2-[(3-phe- nylpropylamino)benzoic acid (NPPB) was introduced at \(t = 30\) min. Ca\(^{2+}\)-free medium or Na\(^{+}\)-deficient medium was introduced at \(t = 20\) min. When the Na\(^{+}\)-deficient medium was used, NaCl was replaced by an iso-osmotic concentration of N-methyl-D-glucamine.

**Neurotransmitter Release Determination**

\[^3H\]D-ASP or \[^3H\]glycine radioactivity was determined in each fraction collected and in the superfused filters by liquid scintillation counting. Endogenous glutamate was determined by high-performance liquid chromatography analysis following precolumn derivatization with o-phthalaldehyde and separation on a C\(_{18}\) reverse-phase chromatographic column (Chrompack, Middelburg, The Netherlands; 10 × 4.6 mm, 3 \(\mu\)m; 30°C) coupled with fluorometric detection (excitation wavelength 350 nm, emission wavelength 450 nm). Buffers and the gradient program were as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/ethanol, 80:20; gradient program, 15% B and 85% C for 1 min from the initiation of the program; 50% B and 50% C in 1 min; 30% B and 70% A in 1 min; 100% B in 0.5 min; isocratic flow 2 min; flow rate 0.9 ml/min. Homoserine was used as internal standard.

**Calculations**

Tritium released in each fraction collected was calculated as fractional rate. The endogenous glutamate release was expressed as picomoles per milligram of synaptosomal protein. Drug effects were evaluated by calculating the ratio between the efflux in the fourth fraction collected (in which the maximum effect of GABA was generally reached) and that of the second fraction. This ratio was compared with the corresponding ratio obtained under control conditions. Appropriate controls were always run in parallel. The concentration-
tion of the basal release. The data presented are mean 
6
chambers for each experimental condition).

four to nine experiments performed in triplicate (three superfusion
ASP (solid circles) and [3H]glycine (open circles) release from mouse
pig antivesicular glutamate transporters of type 1 (vGLUT-1;
rabbit anti-GABA transporters type 1 (GAT-1; 1:50); guinea

containing 3% albumin. The following antibodies were used:
bated 30 min with the primary antibodies diluted in PBS con-
containing 3% serum albumin, the preparations were incu-

end of the second fraction collected and maintained until the end
of the experiment. Fractions were collected and counted for radioactiv-
ity. The release of tritium in the second fraction collected (control
the experiment. Fractions were collected and counted for radioactiv-
tions of GABA. GABA was added to the superfusion medium at the
radioactive tracers and exposed in superfusion to various concentra-
tions of GABA. GABA was added to the superfusion medium at the end of the second fraction collected and maintained until the end of the experiment. Fractions were collected and counted for radioactivity. The release of tritium in the second fraction collected (control basal release) amounted to 3.23% ± 0.15% of total synaptosomal tritium content (n = 9). Results are expressed as percentage potentiation of the basal release. The data presented are mean ± SEM of four to nine experiments performed in triplicate (three superfusion chambers for each experimental condition).

response curve shown in Figure 1 was fitted to the experimental data by using the following four-parameter logistic equation, provided by the software Sigma Plot version 8.0:

\[ y = a + \frac{(b - a)}{1 + (10^c/10^d)^n} \]

where a is the minimum and b the maximum value, c is the EC_{50}, and d is the slope of the curve.

Confocal Microscopy

Synaptosomes (100 μg protein) obtained by means of Percoll gradients (Dunkley et al., 1988) were placed onto coverslips pretreated with poly-L-ornithine and maintained 30 min at 37°C in a 5% CO₂ 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 phosphate-buffered saline (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 antibodies were used: rabbit anti-GABA transporters type 1 (GAT-1; 1:50); guinea pig antivesicular glutamate transporters of type 1 (vGLUT-1; 1:1,000), guinea pig antiglutamate transporter EAAT2 (also named GLT-1; 1:1,000). After washing (3 × 5 min) with PBS containing 0.5% serum albumen, the preparations were incubated for 30 min with the appropriate secondary Alexa Fluor 488- or Cy3-labelled antibodies diluted in PBS containing 3% albumin and washed for 3 × 5 min. Fluorescence image acquisition was performed by a three-channel Leica TCS SP2 laser scanning confocal microscope equipped with 458-, 476-, 514-, 543-, and 633-nm excitation lines. Images (512 × 512 × 8 bit) were taken through a plan-apochromatic oil-immersion objective ×100/NA 1.4. Light collection configuration was optimized according to the combination of chosen fluorochromes, and sequential channel acquisition was performed to avoid cross-talk phenomena. The Leica LCS software package was used for acquisition, storage, and visualization. The on-line Web service “Power Up Your Microscope” (www.powermicroscope.com, Department of Physics, University of Genoa, Italy, and Re@lityNET, Genova, Italy) was employed to perform deconvolution of images, increasing both image resolution and signal-to-noise ratio. Spatial colocalization was analyzed through 2D correlation cytofluorograms accomplished by routines integrated in the Leica LCS software.

Statistical Analysis

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

Drugs

[^3H]D-aspartate (specific activity 16.3 Ci/mmol) and [^3H]glycine (specific activity 10 Ci/mmol) were purchased from Amersham (Buckinghamshire, United Kingdom). GABA, muscimol, niflumic acid, and NPPB were obtained from Sigma Chemical Co. (St. Louis, MO). 5,7-Dichlorokynurenic acid, DL-TBOA, and dihydrokainic acid (DHK) were from Tocris Cookson (Bristol, United Kingdom); BPATa from Fluka Biochemika (Milan, Italy); Percoll from Pharmacia (Uppsala, Sweden); and the anti-PSD-95 monoclonal antibody from Affinity Bioreagents Inc. (Goldenn, CO). Anti-GAT-1 (AB1570(W), anti-vGLUT-1 (AB5905), and anti EAAT-2 (AB1783) primary antibodies were obtained from Chemicon (Temecula, CA). The donkey anti-rabbit Alexa Fluor 488-conjugated secondary antibody (A-21206) was purchased from Molecular Probes Europe (Leiden, The Netherlands). The donkey anti-guinea pig Cy3-conjugated secondary antibody (706-165-148; Jackson Laboratories, Bar Harbor, ME) was a generous gift of Dr. Paola Ramoino (University of Genoa, Genoa, Italy).

RESULTS

We studied the effect of GABA on the release of glutamate from spinal cord nerve terminals. Unlike the case in previous works on transporter coexistence (for review see Raiteri et al., 2002), which were performed with rat brain synaptosomes, we here used nerve endings isolated from mouse spinal cord, also in view of the possible utilization of genetically modified animals. Figure 1 shows that exogenous GABA, added to the superfusion

Fig. 1. Concentration-response curves of the GABA-evoked [^3H]D-ASP (solid circles) and [^3H]glycine (open circles) release from mouse spinal cord synaptosomes. Synaptosomes were labelled with the radioactive tracers and exposed in superfusion to various concentrations of GABA. GABA was added to the superfusion medium at the end of the second fraction collected and maintained until the end of the experiment. Fractions were collected and counted for radioactivity. The release of tritium in the second fraction collected (control basal release) amounted to 3.23% ± 0.15% of total synaptosomal tritium content (n = 9). Results are expressed as percentage potentiation of the basal release. The data presented are mean ± SEM of four to nine experiments performed in triplicate (three superfusion chambers for each experimental condition).
medium, increased the spontaneous release of \([3H]D\)-ASP from synaptosomes prelabeled with the radioactive amino acid. The releasing effect of GABA was concentration dependent: the maximal potentiation of release, reached at 10 μM, was about 100% over basal; the calculated EC50 value amounted to 3.76 μM.

Because the release of \([3H]D\)-ASP represents the response to GABA of the “newly taken up” pool of glutamate, experiments were carried out by monitoring the release of endogenous glutamate. GABA (1 or 10 μM) potentiated by about 40% and 90%, respectively, the release of endogenous glutamate (Fig. 2), suggesting that \([3H]D\)-ASP release parallels that of the endogenous transmitter.

Interestingly, when mouse spinal cord synaptosomes prelabeled with \([3H]\)glycine were exposed to GABA during superfusion, no significant release of tritium could be observed at 10 μM of GABA (Fig. 1), the concentration producing maximal effect on the release of \([3H]D\)-ASP. The release of tritium was increased by ~30% only when GABA was added at 300–1,000 μM.

To identify the target of GABA on the glutamate-releasing terminals, synaptosomes prelabeled with \([3H]D\)-ASP were exposed to the GABA\(_A\) receptor agonist muscimol or to the GABA\(_B\) receptor agonist \((–)\)baclofen. Figure 3 shows that neither muscimol (10 μM) nor \((–)\)baclofen (100 μM) enhanced the basal release of \([3H]D\)-ASP, thus excluding the involvement of GABA receptors. Figure 3 also shows that the release of \([3H]D\)-ASP provoked by 10 μM GABA was largely prevented by the GABA GAT-1 transporter inhibitor SKF89976A, suggesting that the release of \([3H]D\)-ASP is triggered by GABA uptake into glutamatergic axon terminals. The GABA (10 μM)-evoked release of endogenous glutamate also was strongly prevented (~80%) by 10 μM SKF89976A (Fig. 2).

The hypothesis of a glutamate release elicited by GABA uptake is supported by the observation that the GABA-evoked \([3H]D\)-ASP release was strictly dependent on the [Na\(^+\)] present in the superfusion medium. As illustrated in Figure 4, the effect of GABA (10 μM) was inhibited by ~60% when extraterminal [Na\(^+\)] was 46 mM and was abolished in Na\(^+\)-free medium. Figure 2 shows that the release of endogenous glutamate elicited by 10 μM GABA was totally prevented when the [Na\(^+\)] in the superfusion medium was lowered to 23 mM.

The release data strongly suggest that GABA transporters exist on glutamatergic nerve terminals, where they modulate glutamate release (see also Discussion);
however, they do not give information about the proportion of excitatory terminals involved. To estimate the percentage of glutamatergic synaptosomes expressing GABA transporters, confocal microscopy experiments were performed. The results reported in Figure 5i show the GAT-1- or vGLUT-1-positive particles present in the synaptosome preparation; synaptosomes were labelled with anti-GAT-1 (green, Fig. 5iA) and with anti-vGLUT-1 (red, Fig. 5iB) antibodies. The preparation efficiently stained for both GAT-1 and vGLUT-1; many synaptosomes were also efficiently stained for both GAT-1 and vGLUT-1; they constitute no more than a monolayer. Many studies have demonstrated that, when such a synaptosomal layer is up–down superfused, any compound released is removed before it could affect the GABA-evoked [3H]-D-ASP release. Entrapped BAPTA had been found to inhibit the release of glutamate evoked by high K+ (Raiteri et al., 2000). Figure 6 also shows that the effect of GABA (10 μM) was slightly, though significantly, inhibited when DL-TBOA, a nontransportable blocker of glutamate carriers (Shimamoto et al., 1998), was present in the extraterminal medium. The maximal inhibition amounted to ~30% and was obtained by adding DL-TBOA at 10 μM; in fact, 100 μM DL-TBOA was unable to further diminish the releasing effect of 10 μM GABA. Figure 6 also shows that the effect of GABA was reduced by about the same extent by 100 μM DHK, a selective EAAT-2 transporter blocker (Arriza et al., 1994). The effect of GABA on the release of [3H]-D-ASP from mouse spinal cord synaptosomes was strongly reduced by niflumic acid (30 or 300 μM) or NPPB (10 or 100 μM), two anion channel blockers (Fig. 7). Similarly to [3H]-D-ASP release, DL-TBOA (10 μM) and NPPB (100 μM) also prevented the 10 μM GABA-evoked release of endogenous glutamate (not shown).

**DISCUSSION**

The major findings of the present work are that 1) in the mouse spinal cord GABA transporters of the GAT-1 type coexist with glutamate transporters on glutamatergic nerve terminals; 2) activation of GAT-1 transporters enhances spontaneous glutamate release in part by transporter reversal and largely through anion channels; and 3) GABA appears unable to elicit glycine release. The technique used here to monitor release from synaptosomes has been shown to be well suited to identifying targets located on a particular family of nerve terminals. Synaptosomal preparations contain the different nerve ending families present in the mammalian CNS. To study the effects of a compound A on the release of neurotransmitter B, indirect effects have to be minimized. This can be obtained by stratifying synaptosomes on microporous filters (diameter 2.5 cm) in such an amount (<100 μg protein/filter) that, based on previous estimates (Raiteri et al., 1986), the particles constitute no more than a monolayer. Many studies have demonstrated that, when such a synaptosomal layer is up–down superfused, any compound released is removed before it can activate synaptosomal targets (transporters, presynaptic receptors, and so on). These targets, therefore, remain ligand free and can be selectively activated by ligands added to the superfusion medium (Raiteri and Raiteri,
2000). Under such conditions, changes in the release of B produced by A are due to direct action of A on the particles releasing B. For the above-mentioned reasons, superfused synaptosomes permit unequivocal establishment of colocalization of presynaptic targets on a given family of nerve endings and understanding their possible cross-talk (Pittaluga et al., 2000; Raiteri et al., 2001a, 2004; Risso et al., 2004).

Accordingly, the enhancement of the basal glutamate release observed when synaptosomes are exposed to exogenous GABA in superfusion indicates that glutamate-releasing axon terminals in the mouse spinal cord possess targets for GABA. The effect of GABA could not be mimicked by the GABA_A agonist muscimol or the GABA_B agonist (–)baclofen, excluding the involvement of GABA receptors.

The sensitivity of the GABA-evoked glutamate release to SKF 89976A, a GABA transporter inhibitor selective for the GAT-1 type transporter (Borden et al., 1994; Zhou et al., 2004), and the strict Na^+ dependence of the GABA effect indicate that GABA uptake is the primary trigger for glutamate release. Thus, in consideration of the characteristics of the superfusion technique described above, at least some of the axon terminals able...

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**Fig. 5.** Identification by immunocytochemistry of the GABA transporter type 1 (GAT-1), the vesicular glutamate transporter type 1 (vGLUT-1), and the plasma membrane glutamate transporter type 2 (EAAT-2) in synaptosomes purified from mouse spinal cord. Synaptosomes were glued onto coverslips, fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with the primary and secondary antibodies. i: Images show the Alexa Fluor 488-tagged anti-GAT-1 (A) and the Cy3-tagged anti-vGLUT-1 (B). C highlights in yellow the colocalized pixels representing coexpression of GAT-1 and vGLUT-1. Two pixels are considered as colocalized 1) if their respective intensities (0–255, 8 bit) are higher than the threshold of their channels, set to 100 for both green and red channels, and 2) if the ratio of their intensities is strictly higher than 50%. D shows the correlation cytofluorogram of the images in A and B; the white straight lines delimit the area defined by condition 2. Colocalized pixels are displayed in blue. ii: Images show the Alexa Fluor-488 tagged GAT-1 (A) and the Cy3-tagged anti-EAAT-2 (B). Colocalization of pixels is reported in C and the cytofluorogram in D. Colocalization constrains are described above.
to take up \[^{3}H\]D-ASP, and that therefore possess glutamate transporters, coexpress transporters for GABA of the GAT-1 type, the activation of which elevates the basal release of glutamate. In our synaptosomal preparation, GABA provoked glutamate release with an EC\(_{50}\) of 3.76 μM, well in keeping with Km values for high-affinity GABA uptake obtained with various preparations (Levi, 1970; Guastella et al., 1990).

Activation of GAT-1 GABA heterotransporters selectively potentiates the spontaneous release of glutamate but not the release evoked by depolarization. In fact, it was shown that the release of glutamate caused by depolarizing stimuli in spinal cord synaptosomes was depressed by GABA through the activation of GABA\(_B\) heteroreceptors (see Bonanno and Raiteri, 1993).

With regard to the mechanism(s) by which glutamate exits from nerve terminals during GABA uptake, we first considered the two processes most frequently involved in neurotransmitter release: exocytosis and carrier-mediated release. Conventional exocytosis triggered by Ca\(^{2+}\) ions entering through voltage-sensitive Ca\(^{2+}\) channels is not involved in the efflux of glutamate elicited by GABA, because the effect of GABA is independent of external Ca\(^{2+}\). Exocytosis resulting from mobilization of intraterminal Ca\(^{2+}\) from internal stores also seems unlikely to occur, insofar as the GABA-evoked glutamate release was not reduced in synaptosomes containing the Ca\(^{2+}\) chelator BAPTA.

It is well known that, under some conditions, neurotransmitters can be released directly from the cytosol through the homotransporter working in the inside–out direction (see Levi and Raiteri, 1993). This carrier-mediated release is Ca\(^{2+}\) independent and sensitive to homotransporter inhibitors. In this study, we found that the GABA-evoked release of glutamate was in part inhibited by DL-TBOA, a nontransported blocker of glutamate carriers (Shimamoto et al., 1998), indicating that a portion of glutamate release occurs by homotransporter reversal, possibly facilitated by Na\(^{+}\) ions cotransported with GABA by the plasma membrane GABA carriers. The data obtained with DHK suggest that this carrier-mediated release is actuated by glutamate transporters of the EAAT-2 type, expressed on the external membrane of the glutamatergic axon terminals. Interestingly, our release results and confocal microscopy images (see below), obtained in spinal cord, support recent data in the literature showing that the EAAT-2 protein is present in purified synaptosomes as well as in glutamate nerve endings of mature hippocampal neurons, where it recaptures glutamate back into excitatory terminals (Suchak et al., 2003; Chen et al., 2004).

The results obtained with niflumic acid and NPPB suggest that the majority of the glutamate released by GABA exits through anion channels. Different types of Cl\(^{-}\)/anion channels are present on cell membranes.
Among these channels, the volume-sensitive outwardly rectifying anion channels (VSOAC) are ubiquitously expressed. VSOAC are permeable not only to Cl- ions but also to organic osmolytes, including glutamate. Activation of VSOAC can be prevented by nitromycin acid and NPPB (see Pasantes-Morales, 1996; Maduke et al., 2000). Although activation of VSOAC generally occurs during cell swelling caused by strong hypotonicity, evidence exists that some anion channels can also open under near-isotonic conditions. In particular, it was shown that [3H]D-ASP can be released through anion channels in hippocampal slices after small and gradual increases in intracellular osmolarity (Franco et al., 2000). Although a possible effect of NPPB or nitromycin acid on electrophysiological gradients, known to drive transporter activity, could not be completely ruled out, it can be hypothesized that, under our experimental conditions, the entry into spinal cord synaptosomes of Cl- ions cotransported with GABA is sufficient to trigger Cl-/anion channel activation. Interestingly, glutamate had been found to be released through an NPPB-sensitive mechanism from cerebellar parallel fiber terminals/varicosities during activation of Cl- permeable Glutamate Transporter Interaction 431

The release studies revealed uptake of GABA by specific transporters into glutamate-releasing nerve terminals and the consequent efflux of glutamate. Release studies, however, cannot illuminate the proportion of excitatory terminals bearing transporters for GABA. Confocal microscopy experiments performed with synaptosomes purified from mouse spinal cord show that particles positive for vGLUT-1 antibodies are also positive for antibodies anti-GABA transporter of the GAT-1 type and that GAT-1 coexists with the glutamate transporter EAAT-2 in these particles, in even a larger proportion.

These morphological results have different possible explanations. One possibility is that GAT-1 transporters, EAAT-2 transporters, and v-GLUT-1 transporters all exist in the same nerve terminal. Another possibility stems from the well-known existence of postsynaptic membranes often attached to the synaptosomes, suggesting for instance that EAAT-2 and vGLUT-1 are in the terminal bouton, whereas GAT-1 is on the postsynaptic membrane. In the latter case, however, the release of glutamate evoked by GABA transporter activation could not be observed. Thus, the morphological results, when seen in the light of the functional data, can be best interpreted as indicating coexistence of GABA and glutamate transporters on the same nerve terminal. Although the quantitative evaluation of confocal microscopy images of purified synaptosomal fractions requires caution, because of the possible presence of nonsynaptosomal elements, the observation that, under our experimental conditions, about 35% of vGLUT-1 colocalize with GAT1 allows us to conclude that a noteworthy amount of glutamatergic synaptosomes expresses GABA transporters. Interestingly, the percentage of colocalization of GAT-1 and EAAT-2 seems to be even more pronounced than that of GAT-1 and vGLUT-1. This would reflect the presence of EAAT-2 transporters on GABAergic terminals. Indeed, the presence of glutamate transporters, the activation of which leads to GABA release, was reported in different rodent brain regions (Bonanno et al., 1993), including the mouse spinal cord (data not published).

The finding that GABA, added at the concentration that maximally stimulates the release of glutamate, was unable to potentiate the basal release of glycine from mouse spinal cord nerve endings deserves some comment. It is known that, in the spinal cord, some nerve terminal costore GABA and glycine (Burger et al., 1991; Todd et al., 1996), which can be coreleased onto motorneurons (Jonas et al., 1998). Spinal nerve terminals that contain glycine-, GABA- or both neurotransmitters express the same vesicular transporter, termed vesicular inhibitory amino acid transporter (VIAAT; Sagné et al., 1997; Chaudry et al., 1998). We previously found that activation of glycine transporters (GLYT-1 and GLYT-2) on spinal cord synaptosomes mediates release of GABA, in part by exocytosis and in part through reversal of the GABA transporters coexisting with glycine transporters on the same terminals (Raiteri et al., 2001a). The modest effect of high GABA concentrations on the release of glycine (present work) indicates that the abundant “pure” glycineergic terminals do not possess high-affinity GABA transporters able to mediate glycine release. As for the terminals costoring GABA and glycine, the unexpected inability of GABA to evoke glycine release has various possible explanations. One reason is that GABA and glycine might not be stored in the same vesicles, as proposed by some authors (McIntire et al., 1997). Another possibility originates from the kinetic properties of the glycine transporters of the GLYT-2 type, the major homotransporter type on glycineric terminals (Poyatos et al., 1997). It was reported that GLYT-2 has a kinetic constraint for reverse transport that strongly limits glycine release (Roux and Supplisson, 2000; Herdon et al., 2001). Thus, the native GLYT-2 transporters coexisting with GABA transporters on some mouse spinal cord nerve endings (Raiteri et al., 2001a) might blunt glycine release by transporter reversal.

In conclusion, nerve terminals seem to coexpress homo- and hetero transporters also in the absence of cotransmission, i.e., when the corresponding transporter substrates do not coexist in those terminals. On the other hand, coexistence of transporters in case of cotransmission might not necessarily imply reciprocal modulation of cotransmitter release.

The finding that GABA can stimulate the spontaneous release of the major excitatory transmitter glutamate may appear surprising. On the other hand, several examples of depolarizing GABA_A receptors, including GABA_A receptors able to trigger neurotransmitter exocytic release, have been reported (Rohrbaugh and Spitzer, 1996; Staley et al., 1996; Wagner et al., 1997; Fassio et al., 1999). The present results suggest that GABA may stimulate transmitter release by a receptor-independent mechanism, i.e., by activating high-affinity...
GABA GAT-1 heterotransporters localized on spinal cord glutamatergic nerve endings. The process here characterized in the spinal cord might have pathophysiological implications, for instance, in the mechanisms of pain. In a very recent study with synaptosomes isolated from neocortical and hippocampal biopsies of patients with temporal lobe epilepsy, it was found that the functions of GABA and glutamate transporters are differentially impaired (Hoogland et al., 2004). This suggests that differential malfunctioning of GABA and glutamate transporters coexisting on the same neuron can underlie CNS pathologies.

Evidence is accumulating that proteins coexpressed on the same membrane can interact. Although most studies have focused on interactions at the cell body/dendrites level, interactions can also take place on nerve endings between presynaptic receptors (Svensson et al., 2001; Engelmann and MacDermott, 2004; Risso et al., 2004), between presynaptic receptors and transporters (Beckman et al., 1999; Jones et al., 1999; Daws et al., 2004), and between homo- and heterotransporters (present work; for review see Raiteri et al., 2002). It now seems clear that these interactions are rarely direct but involve complex and heterogeneous intracellular pathways, which should be carefully characterized.

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