The sodium- and chloride-coupled glycine neurotransmitter transporters (GLYTs) control the availability of glycine at glycine-mediated synapses. The mainly glial GLYT1 is the key regulator of the glycine levels in glycinergic and glutamatergic pathways, whereas the neuronal GLYT2 is involved in the recycling of synaptic glycine from the inhibitory synaptic cleft. In this study, we report that stimulation of P2Y purinergic receptors with 2-methylthioadenosine 5′-diphosphate in rat brainstem/spinal cord primary neuronal cultures and adult rat synaptosomes leads to the inhibition of GLYT2 and the stimulation of GLYT1 by a paracrine regulation. These effects are mainly mediated by the ADP-prefering subtypes P2Y1 and P2Y13 because the effects are partially reversed by the specific antagonists N6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate and pyridoxal-5′-phosphate-6-azoido(2-chloro-5-nitrophenyl)-2,4-disulfonate and are totally blocked by suramin. P2Y12 receptor is additionally involved in GLYT1 stimulation. Using pharmacological approaches and siRNA-mediated protein knockdown methodology, we elucidate the molecular mechanisms of GLYT regulation. Modulation takes place through a signaling cascade involving phospholipase C activation, inositol 1,4,5-trisphosphate production, intracellular Ca2+ mobilization, protein kinase C stimulation, nitric oxide formation, cyclic guanosine monophosphate production, and protein kinase G-I (PKG-I) activation. GLYT1 and GLYT2 are differentially sensitive to NO/cGMP/PKG-I both in brain-derived preparations and in heterologous systems expressing the recombinant transporters and P2Y1 receptor. Sensitivity to 2-methylthioadenosine 5′-diphosphate by GLYT1 and GLYT2 was abolished by small interfering RNA (siRNA)-mediated knockdown of nitric-oxide synthase. Our data may help define the role of GLYTs in nociception and pain sensitization.

Glycine is the major inhibitory neurotransmitter in the caudal areas of the brain, spinal cord, brain stem, and retina and plays an important role in the processing of sensory, motor, and nociceptive information. The levels of neurotransmitter in glycinergic synapses are controlled by Na+ and Cl−-dependent active transport through the plasma membrane (1). GLYT1 is associated with both glycinergic and glutamatergic pathways and is the main regulator of the glycine levels in the synapses. The neuronal glycine transporter GLYT2 is involved in supplying glycine to the inhibitory nerve terminal to refill synaptic vesicles, and it assists the GLYT1 isomorph in maintaining low synaptic glycine levels (2). Hypofunction of glycine signaling is implicated in neuromotor disorders, nociceptive and neuropathic pain, and epilepsy. Mutations in the GLYT2 gene (SLC6A5) have been identified as the major presynaptic defect causing hyperekplexia or startle disease in humans, as well as congenital muscular dystrophy type 2 in calves (3). Moreover, GLYT1 inhibitors may improve cognitive deficits of schizophrenia by increasing glycine levels around the NMDA3 receptors (4). Analysis of knock-out animals proved that the modulation of glycine transporter expression and/or transport activity influences glycine-mediated neurotransmission and opened a way to find therapeutic applications (5, 6). The levels of active glycine transporters in the plasma membrane are regulated by several mechanisms, including protein trafficking (7–9), Ca2+ modulation (10, 11), protein-protein interaction (11), and membrane raft association (9), among others (7, 12). In the central nervous system (CNS), these regulatory pathways must be triggered by physiological stimuli such as the neural function or the activity of appropriate receptors.

The P2 purine nucleotide receptors respond preferentially to ATP and include ionotropic (P2X) and metabotropic (P2Y) types (13). P2X are ATP-gated cation channels that allow the permeation of Ca2+, Na+, and K+ upon stimulation. The P2Y receptors (P2YR) are G protein-coupled receptors that may regulate two important second messengers cytoplasmic Ca2+ and cyclic adenosine monophosphate (cAMP), depending on

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3 The abbreviations used are: NMDA, N-methyl d-aspartate; CPTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; GFAP, glial fibrillary acidic protein; GLYT, glycine transporter; MCEADP, 2-methylthio-adenosine-5′-diphosphate; MRS2179, 7′-methyl-2′-deoxyadenosine-3′,5′-bisphosphate; MRS2211, pyridoxal-5′-phosphate-6-azo(2-chloro-5-nitrophenyl)-2,4-disulfonate; NFPS, N-[3-(4-fluorophenyl)-propyl]sarcosine; P2R, purinergic receptor; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; SNP, sodium nitroprusside; PLC, phospholipase C; DIV, days in vitro; eNOS, endothelial NOS; nNOS, neuronal NOS; P2YR, P2Y receptor; BAEC, Bovine aortic endothelial cell.
the particular subtype (14). The seven mammalian P2X receptor subunits (P2X<sub>1</sub>–<sub>7</sub>) and eight mammalian P2YRs (P2Y<sub>1,2,4,6,11,12,13,14</sub>) so far cloned (15, 16) are widely expressed in the mammalian CNS, and their activation generally results in excitatory neuronal responses (17). Purinergic signaling modulates extracellular neurotransmitter levels (acetylcholine, the monoamines, glutamate, GABA, adenosine, or glycine) (16, 18, 19). ATP released from neurons or glial cells has a major role in the immune and neuroinflammatory events involving neuron-microglia intercellular communication, such as neuropathic pain (14). In addition, P2 receptors have been involved in the generation or modulation of nociceptive signals (20, 21).

Glycine neurotransmitter transporters are abundant in glycineergic pathways of the spinal cord dorsal horn, a region containing P2R, and play an important role in the regulation of pain signal transduction (22). A loss of GABAergic and glycineergic synaptic inhibition in this region has been recognized as an important process in the development and maintenance of chronic pain (23, 24). Pharmacological blockade of glycine transporters in the spinal cord increases either glycineergic or GABAergic neurotransmission in vivo and produces pain relief in mouse pain models (25–30). This suggests that glycine transport can modulate the function of glycineergic dorsal horn neurons. This fact prompted us to study whether GLYTs could be targets of purinergic regulation. In this study, we demonstrate that the stimulation of P2 receptors in rat brainstem primary neuronal cultures, and adult rat synaptosomes, modulate GLYTs transport activity by a paracrine mechanism involving nitric oxide production and protein kinase G (PKG) activation. Remarkably, the modulation of GLYTs by purinergic signaling was opposite for GLYT2 and GLYT1, leading to inhibition of the neuronal transporter but stimulation of GLYT1. Our data may help define the role of GLYTs in nociception and brain function.

**Experimental Procedures**

**Materials**—Wistar rats were bred at the Centro de Biología Molecular Severo Ochoa (Madrid, Spain). The experiments were performed in accordance with the Royal Decree 1201/2005 of the Spanish Ministry of Presidency for the protection of animals used in scientific research. [3H]Glycine (1.6 TBq/mmol) was purchased from PerkinElmer Life Sciences, and 2-methylthioadenosine 5′-diphosphate (2MeSADP), suramine, N<sup>6</sup>-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2179), pyridoxal-5′-phosphate-6-azo(2-chloro-5-nitrophenyl)-2,4-disulfonate (MRS2211), and TNP-ATP were from Sigma. Cangrelor (AR-C99931MX) was a generous gift of The Medicines Co., Parsippany, NJ. Antibodies were from the following sources: P2 receptors from Alomone; MAP2 and GFAP from Sigma. Goat GLYT1 antibody was from Chemicon (Millipore). Mouse eNOS antibody was from BD Transduction Laboratories. Secondary antibodies coupled to fluorophores were from Molecular Probes. Rabbit and rat antibodies against the GLYT2 N terminus have been previously characterized (9, 31). The GLYT2 inhibitor ORG25543 was generously donated by Dr. Zoran Rankovic, Organon, Scotland, United Kingdom (32). The GLYT1 inhibitor N′-[3-(4-fluorophenyl)-3-(4-phenylphe-noxy)-propyl]sarcosine (NFPS) was a gift of Dr. Jesús Benavides (Sanofi-Aventis, Vitry sur Seine, France). All other reagents were obtained from Sigma.

**Neuron-enriched Brainstem Primary Cultures**—Brainstem primary cultures were performed as described previously (33) with modifications. Brainstems and spinal cords from 16-day-old rat fetuses were isolated in Hanks’ balanced salt solution (Invitrogen) and dissociated with 0.25% trypsin (Invitrogen) and 4 mg/ml DNase (Sigma). Cells were incubated for 4 h in plating buffer (Dulbecco’s modified Eagle’s medium, Invitrogen, containing 10% fetal calf serum and supplemented with 10 mM glucose, 10 mM sodium pyruvate, 0.5 mM glutamine, 0.05 mg/ml gentamicin, 0.01% streptomycin, 100 microns/ml penicillin G), and buffer was then replaced by culture medium (Neurobasal/B27 50:1 by volume, Invitrogen, containing 0.5 mM glutamine). At 2 days in vitro (DIV) cytosine arabinoside was added to a 10 μM final concentration and was progressively diluted with fresh medium added every 5 days until the cultures were used (12–15 DIV). Cells were plated on polylysine (13 μg/ml)-coated 24-well plates at a density of 200,000 cells/well (for transport assays) or 40,000 cells/well on coverslips (immunofluorescence).

**Isolation of Brainstem Synaptosomes and Pharmacological Treatments**—Synaptosomes were purified from rat (3 months old) brainstem and spinal cord as described previously (10). Purified synaptosomes were incubated in HEPES-buffered medium (HB, composition in mM: NaCl 140, KCl 5, MgCl<sub>2</sub> 1, Na<sub>2</sub>HPO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 5, glucose 10, and HEPES-NaOH 20, pH 7.4) at 37 °C for 5 min with vehicle or the indicated P2 antagonist: suramine (100 μM), MRS2179 (20 μM), or MRS2211 (20 μM), and additionally incubated for 5 min with the P2 agonist 2MeSADP (0.1–10 μM). Subsequently, glycine transport was determined as indicated below. Pharmacological treatments on brainstem primary neuronal cultures were performed as described for synaptosomes but using PBS (composition in mM: NaCl 137, Na<sub>2</sub>HPO<sub>4</sub> 8, KH<sub>2</sub>PO<sub>4</sub> 1.8, KCl 2.6, CaCl<sub>2</sub> 1, MgSO<sub>4</sub> 0.4, glucose 10, pH 7.2) instead of HB.

**Glycine Transport Assays**—Uptake assays in brainstem primary neuronal cultures were performed at 37 °C in PBS as described previously (8) with modifications. For GLYT2 activity determination, uptake solution contained 2 μCi/ml [3H]glycine (1.6 TBq/mmol; PerkinElmer Life Sciences), cold glycine (10 μM final concentration) plus 5 μM NFPS to inhibit glycine transport by GLYT1 (IC<sub>50</sub> = 16 nM) with or without the specific GLYT2 inhibitor ORG25543 (1 μM, IC<sub>50</sub> = 30 nM) or ALX1393 (0.5 μM, IC<sub>50</sub> = 50 nM) to measure background glycine accumulation. For GLYT1 activity determination, uptake solution contained the radioactive substrate plus a GLYT2 inhibitor (1 μM ORG25543 or 0.5 μM ALX1393) with or without 5 μM NFPS. GLYT2 activity was totally resistant to NFPS (34), and GLYT1 was totally resistant to the used ORG25543 or ALX1393 concentrations. Aliquots were obtained for scintillation counting and protein quantification (Bradford method). Glycine transport in synaptosomes was performed for 10 min at 37 °C as described previously (10) in HEPES-buffered medium containing 2 μCi/ml [3H]glycine.

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and 1 μM final glycine concentration. Synaptosomes were filtered through 0.45-μm nitrocellulose filters (Millipore), and the [³H]glycine content was determined. In both primary neuronal cultures and synaptosomes, background glycine accumulation was measured in the presence of the GLYT1 or GLYT2 inhibitor and was subtracted to the total glycine transport so that GLYT-mediated glycine transport was defined as the transport sensitive to the specific inhibitor. Glycine transport in COS7 and BAEC cells was measured as described previously (8) using the specific GLYT1 or GLYT2 inhibitors to measure background glycine transport, which was subtracted from the total glycine transport as described above. All assays were performed in triplicate and expressed as mean ± S.E. Representative experiments are shown that were repeated at least three times with the same results.

**Surface Labeling with Sulfo-NHS-SS-biotin**—Brainstem primary cultures were washed at 4 °C with 1.0 ml of PBS and incubated for 40 min with 1 mg/ml sulfo-NHS-SS-biotin in PBS. Cells were washed three times with 1 ml of the same solution containing 0.25% Triton X-100, 1% SDS, and 0.25% sodium deoxycholate in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.4 mM phenylmethylsulfonyl fluoride (PMSF) plus protease inhibitor mixture (Sigma). Total proteins (T) were solubilized with 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% SDS, and 0.25% sodium deoxycholate in 50 mM Tris, pH 7.4, containing 0.4 mM PMSF and protease inhibitors for 30 min at 4 °C. 40 μl of streptavidin-agarose beads (Sigma) were added per sample and incubated for 3 h at 4 °C with agitation. Bead-bound biotinylated proteins (B) were eluted with Laemml buffer (40 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 mM dithiotherol (DTT), 0.01% bromphenol blue) for 10 min at 70 °C. 7 μg of total protein (T) and 14 μg of biotinylated protein (B) were run on a 7.5% SDS-polyacrylamide gel and Western-blotted using specific GLYT1 and GLYT2 antibodies (35). Bands were visualized with the ECL detection method and quantified on a GS-710 calibrated imaging densitometer from Bio-Rad with Quantity One software by using film exposures in the linear range. Calnexin immunoreactivity was used as a loading control. Standard errors were calculated after densitometry from at least three different experiments.

**Immunofluorescence of Brainstem Primary Neuronal Cultures**—Immunofluorescence in neuron-enriched cultures was performed as reported (36) with modifications. Brainstem primary cells grown on coverslips were fixed with ice-cold methanol or 4% paraformaldehyde and permeabilized with 0.25% Triton X-100, and nonspecific binding sites were blocked with 10% BSA in PBS. Cells were then incubated with the GLYT2 or GLYT1 antibody (1:500–1:2000) together with the desired combination of primary antibodies against purinergic receptors or marker proteins (MAP2 and GFAP) in the absence or presence of antigen peptides. Secondary antibodies were anti-rabbit, anti-rat, or anti-goat antibodies coupled to Alexa Fluor® 555 fluorophore for GLYT2, anti-rabbit antibodies coupled to Alexa Fluor® 488 for P2 receptors, and anti-mouse antibodies coupled to Alexa Fluor® 647 for MAP2 and GFAP. The cells were visualized by confocal microscopy on an LSM 510 confocal microscope (Zeiss) using a vertical microscope Axio Imager.Z1 M (Zeiss).

**Immunofluorescence of Brainstem Synaptosomes**—Purified brainstem-spinal cord synaptosomes were subjected to double immunofluorescence as reported previously (37) using primary antibodies against GLYT2 and purinergic receptors. Samples were visualized in a confocal microscope as described above.

**Dual Immunofluorescence of Tissue Slices**—Adult Wistar rats were deeply anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and transcardially perfused with a fixative solution containing 4% paraformaldehyde in PBS, at a flow rate of 20 ml/min. The brainstem and spinal cord were extracted and maintained overnight in fixative. After washing in PBS, tissue was cut with a vibratome into 50-μm slices that were stored in PBS with 0.02% azide for a maximum of 3 weeks. Dual immunofluorescence in slices was performed as described previously (38), and samples were visualized as above.

**cDNA Subcloning and Expression**—GLYT1 (39) and GLYT2 (40) cDNAs were subcloned into pcDNA3, as described previously. Substitution mutants were generated by site-directed mutagenesis with the QuickChange site-directed mutagenesis kit (Stratagene), using the rat GLYT1 or GLYT2 in pcDNA3 as templates. Two independent Escherichia coli colonies carrying the mutant plasmids were characterized by sequencing and transport activity. Bu5-a10 IMAGE CLONE for P2Y1 receptor was purchased from Geneservice, EcoRI and Xhol sites were introduced by PCR and subcloned into EcoRI and Xhol sites of pcDNA3. Transient expression in COS7 cells was performed as described previously (7) but using Neofectin™ (MidAtlantic Biolabs) following the manufacturer’s protocol. Reproducible results were obtained with 60–70% confluent cells on a 100-mm dish using 8 μg of total DNA. Cells were incubated for 48 h at 37 °C until used. For co-transfection experiments, a 2:1 cDNA ratio for GLYT and P2Y1 receptor was used.

**siRNA Design and Transfection**—BAEC were generously provided by Dr. Santiago Lamas (Centro de Biologia Molecular Severo Ochoa, Madrid) and maintained in culture in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with fetal bovine serum (10%, v/v) as described previously (41). Cells were plated onto 0.2% gelatin-coated culture dishes and studied prior to cell confluence between passages 5 and 9. eNOS siRNA duplexes obtained from Dr. Santiago Lamas (CBMSO, Madrid, Spain). A nonspecific control siRNA was used as a negative control (5-AUU CUG UUA AUC AUU CdtTdT-3; Ensembl Transcript ID ENSBTAT0000007246), designed as described previously (42), were purchased from Ambion (Austin, TX) and obtained from Dr. Santiago Lamas (CBMSO, Madrid, Spain). A nonspecific control siRNA was used as a negative control (5-AUU GUA UGC GAU CGC AGA CdtTdT-3). BAEC were transfected with siRNA with Lipofectamine™ 2000 (Invitrogen), following the manufacturer’s protocol and analyzed 48 h after transfection.

**RESULTS**

**Effect of P2Y Receptor Activation on Glycine Transport by GLYT2**—The isotoxic and selective agonist of the P2Y1, P2Y12, and P2Y13 receptor subtypes, 2MeSADP (43), was used to stimulate these P2YR in neuron rat brainstem primary cultures (Fig. 1A). Around 35% decrease in glycine transport activity by GLYT2 was detected. P2YR antagonist application prior to the agonist addition could reverse this inhibition. The highly selective P2Y1 antagonist, MRS2179, and the P2Y13 antagonist,
MRS2211, partially restored the glycine uptake. However, the P2Y12 antagonist cangrelor failed to restore the transport activity. These results suggest GLYT2 is inhibited by activation of P2Y1R and P2Y13R. As expected, the general P2R antagonist suramin (100 μM) abolished the transport inhibition. This compound also produced a small stimulation, suggesting that GLYT2 may be subjected to a basal inhibition by purinergic receptors stimulated by endogenous ATP. The same GLYT2 inhibition was observed in rat brainstem-spinal cord synaptosomes in response to 2MeSADP (data not shown). Because a cross-talk regulation between P2Y and P2X receptors has been reported previously (44), and the possibility exists that the observed effects of 2MeSADP could be carried out by P2X activation induced by P2YR, we used TNP-ATP, which can act as a general P2X antagonist at 100 μM concentration. Pretreatment with TNP-ATP did not affect the observed inhibition suggesting that P2XR are not needed for GLYT2 inhibition by 2MeSADP. Fig. 1, B and C, shows that time and dose dependence of GLYT2 inhibition by 2MeSADP was also consistent with the reported P2YR features (45, 46). Inhibition was maximal at the shortest assayed time of 1 min and was lost within 10 min. Accordingly, a moderately low concentration of agonist (1

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**FIGURE 1. Effect of P2Y receptor activation on glycine transport by GLYT2.** Primary neuronal cultures were incubated for 5 min at 37 °C in presence of vehicle (water) or 10 μM MRS2179 (A and D), 10 μM MRS2211 (A), 0.5 μM cangrelor (A), 100 μM TNP-ATP (A), or 100 μM suramine (A) followed by a 5-min incubation (A and D) or the indicated time (B and C) with 1 μM (A, B, and D) or 0.5–10 μM (C) 2MeSADP (2 MeS). After washing, glycine uptake by GLYT2 was measured during 10 min at 10 μM glycine (A–C, 100% glycine transport by GLYT2 was 175 pmol of Gly/mg of protein/10 min) or the indicated final glycine concentration (D). V_max are given in nanomoles of Gly/mg of protein/10 min, and K_m values are given in micromolar. E, primary neuronal cultures were treated with vehicle (water) or 1 μM 2MeSADP (2 MeS) and subjected to NHS-SS-biotinylation as described under “Experimental Procedures.” 7 μg of total proteins [T] and 14 μg of surface biotinylated proteins [B] were subjected to Western blot for GLYT2 detection and membranes reprobed for calnexin immunoreactivity (CNX) as loading control. Lower panel in E, densitometric analysis of the average values of three independent Western blots. *, significantly different from control, p < 0.05 by analysis of variance with Dunnett’s post hoc test.
produced the highest inhibition, thus suggesting a rapid receptor desensitization, in agreement with the desensitization properties of P2YRs (47). As reported for other receptors, including P2Y13 (48, 49), a bell-shaped curve was observed for the agonist dose response. This behavior has been explained as the result of promiscuous receptor coupling to different G proteins leading to opposite effects (48). Kinetic parameters of GLYT2 glycine transport were also affected by 2MeSADP (Fig.
showing about 40% reduction of \( V_{\text{max}} \) and a nonrelevant reduction in \( K_m \) values. This was partially reversed by pretreatment with the P2Y1 antagonist MRS2179 (Fig. 1D) and the P2Y13 antagonist MRS2211 (data not shown). No alteration in the amount of surface transporter was detected, as assessed by biotinylation and GLYT2 immunodetection after treatment with vehicle or 2MeSADP, suggesting that P2YR stimulation does not alter GLYT2 trafficking but only transport function (Fig. 1E).

**Localization of GLYT2 and P2Y Receptors**—As a first step in the characterization of GLYT2 inhibition by P2YR stimulation, we determined the relative distribution of transporter and receptors in our experimental system. For this purpose, we used the commercially available P2YR antibodies, which have been extensively employed to establish P2R localization in different brain areas (50, 51). We confirmed that P2YR antibodies specifically recognized the immunoreactive receptors in neuronal cultures and transfected COS cells (see supplemental Fig. S1) and performed multiple immunofluorescence using these antibodies and a GLYT2 antibody we previously characterized (Fig. 2) (9). The majority of GLYT2 immunoreactivity did not co-localize with that of P2Y1R or P2Y13R. Primary neuronal cultures showed no significant fluorescence overlapping (Fig. 2, A and C). In synaptosomes, appearing as red (GLYT2) or green (P2YR) dots, less than 5% fluorescence overlapping indicated the presence of either glycnergic or purinergic synaptosomes rather than mixed terminals (Fig. 2B). Although GLYT2 and P2Y were generally not detected in the same cells, cells expressing one or the other protein were in very close proximity so that terminals containing GLYT2 surrounded the cells containing P2YR. The P2YR-containing cells were neurons (immunopositive to MAP2) but also astrocytes (immunopositive to GFAP), which survived the cytosine arabinoside treatment performed at the 2nd DIV (Fig. 2C). The shown distribution of GLYT2 and P2YR suggests that GLYT2 could be regulated by intercellular signaling mediated by P2YR. However, to confirm that this relative localization was not due to the experimental manipulation needed to obtain the in vitro preparations, we performed dual immunofluorescence for GLYT2 and P2YR on spinal cord slices (Fig. 3). In the nervous tissue P2YR antibodies labeled different subpopulations of neurons with differing intensity and showed a characteristic staining pattern detected with these and other P2YR antibodies (51, 52). In control experiments, no signal was observed when the antigen peptide was included in the staining (data not shown). Again, the GLYT2 label in the slices (Fig. 3, red) did not generally overlap with P2YR immunofluorescence (green), but the GLYT2 and P2YR labels were in close proximity. This was more evident in the ventral horn motoneurons that were intensely stained for P2YR and were totally surrounded by glycnergic terminals containing GLYT2 (Fig. 3A). The very low co-localization was also visible in the dorsal horn (Fig. 3B). Taken together, localization data strongly suggest that GLYT2 is subjected to a paracrine regulation, i.e., involving cell to cell communication.

**Signal Transduction Pathways Involved in the Regulation of GLYT2 Activity by P2Y Receptors**—The biological effects of P2YR activation depend on how they couple to downstream signaling pathways, either via \( G_i \), \( G_{q/11} \), or \( G_s \) proteins. P2Y1,2,4,6,11,13 receptors induce activation of phospholipase C (PLC), whereas P2Y12,13,14 are coupled to the adenylate cyclase system. To explore the signal transduction pathways involved in GLYT2 inhibition by P2YR, we pretreated primary neuronal cultures with different inhibitors of key proteins of the possible cascades (Fig. 4A). Inhibition of protein kinase A (PKA) with H89 permitted the glycine transport inhibition, although pretreatment of primary neuronal cultures with a PLC uncoupler (U73122), or with inhibitors of protein kinase C (PKC, i.e., chelerythrine), calmodulin (W7), or nitric-oxide synthase (nNOS, S-methyl-L-thio-citrulline) prevented GLYT2 inhibition by 2MeSADP. These data are consistent with the P2YR effects mediated by an activation of PLC, leading to the production of inositol 1,4,5-trisphosphate and to the mobilization of intracellular Ca\(^{2+}\) resulting in the stimulation of a variety of signaling pathways such as PKC, as well as NOS and subsequent NO formation. This signaling pathway has been previously reported for P2YR (53–55). To confirm whether the modulation of the GLYT2 activity by P2YR involved the production of nitric oxide, we first checked the sensitivity of GLYT2 activity to the cellular NO levels. Fig. 4, B and C, shows that addition of sodium nitroprusside (SNP), a compound able to generate NO upon interaction with cellular reducing agents, inhibited transport by GLYT2 in a dose-response manner, and this inhibition could be abolished by carboxy-2-phenyl-4,4,5,5-
tetramethylimidazol-1-oxyl-3-oxide (CPTIO), a NO scavenger. The inhibition by SNP decreased with the incubation time and was lost within 4–5 min (Fig. 4C). Although SNP half-life has not been reported in the literature, it is presumed that it can release small NO concentrations during long periods of time. Indeed, long lasting increases in cGMP for up to 1 h have been measured upon SNP treatment (56). Because our SNP solutions were made fresh for each experiment, our data are consistent with the NO half-life of seconds and a subsequent desensitization of the pathway within minutes. It should be noted that the time course of GLYT2 inhibition by SNP and by 2MeSADP was very similar (Fig. 1B), thus suggesting the participation of a common agent in both events. Interestingly, the inhibition of GLYT2 by 2MeSADP was also reversed by CPTIO, reinforcing the involvement of NO production in GLYT2 modulation by P2YR (Fig. 4D). Because CTPIO is membrane-impermeable, our data suggest that GLYT2 inhibition by 2MeSADP is prevented by removal of extracellular NO, in agreement with the well established role of NO as extracellular messenger (57). NO-sensitive guanylyl cyclases are the most important receptors for the signaling molecule NO, and the NO-cGMP signaling pathway is present in neurons of the spinal cord (58, 59). In addition, cGMP signaling is transmitted through the activation of several effector molecules, with protein kinase G-I (PKG-I) the most common target. The highly selective and irreversible soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), or 0.3 μM DT-2 followed by application of vehicle or 1 μM 2MeSADP (2 MeS) for a total of 10 min. GLYT2 transport was measured for 10 min. *, significantly different from vehicle, p < 0.05, and **, p < 0.01 by Student’s t test.
with a selective peptide-based inhibitor of PKG-Iα (DT-2) prevented the decrease in glycine transport induced by 2MeSADP. Basal GLYT2 activity was not affected by these two inhibitors. These findings further link GLYT2 regulation to NOS stimulation and cGMP production and indicate that guanylyl cyclase and PKG are required for GLYT2 inhibition by P2YRs.

**Effect of P2Y Receptor Activation on Glycine Transport by GLYT1**—Short term down-regulation of GLYT2 might produce a transient increase of the inhibitory glycinergic neurotransmission due to increased levels of glycine in the synaptic cleft (61). Synaptic glycine is a substrate of the mostly glial GLYT1 transporter, which is the main regulator of the levels of glycine in glycinergic as well as NMDA receptor-mediated glutamatergic synapses (5). Because the physiological consequences of GLYT2 inhibition by P2YR could be modulated by the activity of GLYT1, we measured the effect of P2YR stimulation on glycine transport by this glycine transporter (Fig. 5). In contrast to the effect on GLYT2, addition of 2MeSADP to primary neuronal cultures produced about a 40% increase in glycine transport activity by GLYT1 that could be reversed by previous application of P2Y1, P2Y12, and P2Y13 antagonists (data not shown). These data suggest that P2Y12Rs are also involved in GLYT1 activation. Again, biotinylation of primary cultures and GLYT1 immunodetection after treatment with vehicle or 2MeSADP showed no alteration in the amount of surface transporter, indicating that P2YR stimulation affects transport function and not GLYT1 trafficking (Fig. 5B, lower panel) as shown for GLYT2. Apparently, the same signal transduction pathways involved in GLYT2 inhibition are also implicated in GLYT1 stimulation by P2YR, including PLC activation, inositol 1,4,5-trisphosphate production, intracellular Ca²⁺ mobilization, stimulation of PKC, NO formation, cGMP production, and PKG activation (Fig. 5, C and D). The only exception was the presumed participation of PKA because H89 blocked the effect. This might be related to the involvement of P2Y12R/P2Y13R. The fact that stimulation was blocked by CTPIO supports NO reaches GLYT1-containing cells from the extracellular milieu (57). Therefore, GLYT1 seems to be sensitive to the cellular NO levels in a differential manner as it was GLYT2 (see SNP action in Fig. 5D).

**Localization of GLYT1 and P2Y Receptors**—We determined the relative distribution of GLYT1 and P2YR in the primary...
neuronal cultures by multiple immunocytochemistry (Fig. 6).

As reported in the literature, GLYT1 is mainly present in glial cells (31), and it has also been detected, although in lower amounts, in glutamatergic neurons (62). In agreement with this localization, we found GLYT1 immunoreactivity both in astrocytes and in neurons, as assessed by detection of cellular markers as GFAP for astrocytes and MAP2 for neurons. Because astrocytes are scarce in our primary cultures (less than 15%), the main location of GLYT1 in our experimental system is neurons. Interestingly, the transporter co-localized with the three assayed P2YR subtypes P2Y1, P2Y12, and P2Y13 in both cell types (Fig. 6 and data not shown).

**Co-expression of Recombinant GLYT1 or GLYT2 with P2Y1 Receptors and eNOS Knockdown**—To confirm the differential regulation of the two glycine transporters by P2Y1R, we co-expressed recombinant GLYT1 or GLYT2 with recombinant P2Y1R in COS7 cells, and we treated the cells with vehicle or 2MeSADP (Fig. 7A). As shown in brain preparations, receptor activation oppositely modulated transport activity by the GLYTs so that GLYT2 was inhibited and GLYT1 was stimulated, and these effects were slightly more pronounced in COS7 cells than in primary neuronal cultures. The effects were reversed by the NOS inhibitor L-nitro-L-arginine methyl ester and mimetized by the cell-permeable cGMP analog 8-Br-cGMP and the NO donor SNP, confirming the involvement of the NO-PKG pathway also in the heterologous system. We confirmed the paracrine nature of the glycine transporter regulation by P2Y1R because the modulation of glycine transport by 2MeSADP was detected not only when transporter and receptor were co-expressed in the same cell but also when GLYTs and P2Y1R were present in different cell populations (Fig. 7B). To further sustain the involvement of the NO pathway in the regulation of GLYT1 and GLYT2 activity by 2MeSADP, we used a nonpharmacological approach consisting of the siRNA-mediated knockdown of nitric-oxide synthase (Fig. 7, C and D). For this purpose, we used BAEC cells in which the expression levels of eNOS were sufficient to be detected by Western blot, a condition that was not attained in COS cells. BAEC were transfected with duplex siRNAs control or specific for eNOS (see “Experimental Procedures”) together with the cDNAs of P2Y1R together with GLYT1 (Fig. 7C) or GLYT2 (Fig. 7D), and the effect of 2MeSADP was determined. About 90–95% depletion of eNOS protein was confirmed by immunoblotting. As shown, sensitivity to 2MeSADP by GLYT1 and GLYT2 was abolished by small interfering RNA-mediated knockdown of
eNOS, thus confirming the involvement of the pathway in the GLYT modulation.

**Target Residues of GLYT modulation by P2Y1 Receptors**

The GLYT{s} belong to the SLC6 family of neurotransmitter transporters together with the GABA and the monoamines: serotonin, dopamine, and norepinephrine transporters (63). Regulation of the transport activity by protein kinase G has been reported to be controlled by homologous serine residues present in the N-terminal portion of the fifth transmembrane domain (64, 65), a cytoplasmic protein region located within or near by the permeation pathway of these 12 transmembrane domain proteins (63). The homologous serine residue in GLYT2 is Ser-420, which lies close to a protein kinase C regulatory region (7). In a previous work, we generated mutant S420A in GLYT2 and showed that it has similar properties than wild type, although the replacement by Glu produced a PKC-resistant transporter (7). To test the ability of the homologous serine residues in the GLYT modulation by P2Y1R, we constructed the homologous mutant in GLYT1 (S244A) and characterized the transport activity of the mutants expressed in COS7 cells (Fig. 8, A and B). Serine mutants were almost equally active as wild types showing about 80–90% V_{max} for glycine transport and a nonsignificantly increased K_{m} value. However, when mutants were co-expressed with P2Y1R, they were insensitive to the modulation by 2MeSADP, in contrast to their corresponding wild types. This points to the homologous serines 244 and 420 as potential target residues of the GLYT regulation by P2Y1R.

**DISCUSSION**

In this work, we report that P2Y receptors exert an opposite modulation of the glycine neurotransmitter transport mediated by the plasma membrane transporters GLYT1 and GLYT2 in brain-derived preparations. GLYT1 is largely present in astro-
Regulation of GLYT1 and GLYT2 by P2Y Receptors

cytos and is the main regulator of the glycine levels in glyciner-gic synapses containing glycine receptors and glutamatergic synapses containing NMDA receptors (5, 31, 66). In addition to its glial localization, GLYT1 has been found in glutamatergic neurons, although in low abundance (38). The neuronal transporter GLYT2 is involved in the recycling of synaptic glycine in glycineric pathways, so that it removes glycine from the synaptic cleft, and hence it preserves the quantal glycine content inside inhibitory synaptic vesicles (6). The activation of P2YR in neuronal primary cultures and synaptosomes leads to the inhibition of the neuronal GLYT2 and the stimulation of GLYT1. These effects seem to be mediated by the ADP-prefering receptors P2Y1 and P2Y13 (and also P2Y12 for GLYT1), because subtype-specific antagonists partially prevented the transporter modulations, and general P2R antagonists completely blocked the effects. Our localization data indicate the presence of these receptors in brainstem astrocytes as well as glutamatergic neurons, in agreement with previous distribution studies (67, 68). P2YR activation induces nNOS translocation to the plasma membrane in PC12 cells, and the implicated pathway required Ca2⁺ mobilization and PKC activation (71). nNOS has been largely found in astrocytes and neurons, and its synthetase activity is regulated by PKC phosphorylation (72). Our data are consistent with the purinergic cells producing and releasing NO to the extracellular compartment. External NO, easily diffusing through cell membranes, gets into the cell interior of neighboring neurons and glial cells and increases the level of the second messenger cGMP and subsequently activates PKG-I (57). The fact that modulations were observed even when GLYT1 and GLYT2 were expressed in separate cell populations, as well as the sensitivity of the modulations to the membrane-impermeable NO scavenger CTPIO, reinforces this hypothesis.

GLYT1 and GLYT2 are differentially sensitive to NO/cGMP/PKG-I both in brain-derived preparations and in heterologous cells expressing the recombinant transporters, indicating that the observed glycine transport modulation is indeed the consequence of specific effects on the transporters. This has been assessed by pharmacological and siRNA-mediated knockdown tools. Other members of the SLC6 transporter family are also

FIGURE 8. Effect of P2Y1R activation on glycine transport by wild type or mutant GLYT1 transporters. A and B, kinetic characterization of S422A (GLYT1) and S420A (GLYT2) mutants. COS cells expressing the indicated wild type or mutant transporters were assayed for glycine transport in the presence of the indicated final glycine concentrations. Vmax are given in nanomoles of Gly/mg of protein/5 min, and Km values are given in micromolar. C, COS7 cells co-expressing P2Y1R together with the indicated wild type or mutant transporters were treated for 5 min with vehicle or 1 μM 2MeSADP, and glycine transport by GLYT1 or by GLYT2 was measured after washing. 100% transport activities were in nanomoles of glycine/mg of protein/5 min: 3.4 (GLYT1), 1.0 (GLYT2), 2.8 (S244A), and 0.82 (S420A). *, significantly different from vehicle, p < 0.05 by Student’s t test.
regulated by the NO/cGMP/PKG-1 pathway (65, 73). The differential regulation of GLYT1 and GLYT2 may involve dissimilar effects of transporter phosphorylation by PKG-1 on homologous TM5 serines (Ser-244 and Ser-420 for GLYT1 and GLYT2, respectively) or interaction of the two transporters with other phosphorylation targets of this kinase, which may trigger differential regulatory effects. Alternatively, the combined effects of PKG-1 and PKA on GLYT1 activity may result in activation instead of inhibition of the transport. The rise in GLYT1 transport by NO may explain the finding that GLYT1 activity has been reported as essential for glycine-mediated protection of human intestinal epithelial cells against oxidative damage (74). This issue deserves future research.

Changes in the balance between local excitatory and inhibitory synaptic inputs in the spinal dorsal horn, and therefore a net increase in spinal excitation is a crucial mechanism in the development and maintenance of chronic pain of both inflammatory and neuropathic origin (22). The glycineric interneurons are localized between axons from primary nociceptive afferents and central projection neurons, and glycineric neurotransmission plays a pivotal role in a variety of pain states (75). The glycine-receptor antagonist strychnine elicits mechanical allodynia that is caused by glycineric disinhibition in the spinal dorsal horn (25). During intense nociceptive input, glycine released from inhibitory interneurons can escape the synaptic cleft and reach adjacent NMDA receptors by spillover, hence facilitating excitatory neurotransmission. Suppression of this process can also induce analgesia (76). Therefore, it has been reported that manipulation of the synaptic glycine concentrations affects nociception by influencing both excitatory and inhibitory transmission. Pharmacological blockade of GLYT2 in the spinal cord increases glycineric neurotransmission in vivo and produces pain relief in rat acute pain models (28) and neuropathic pain models (25, 27, 29, 30). However, inhibition of GLYT1 can enhance both excitatory and inhibitory transmission, and its pharmacological blockade may evoke anti- or pro-nociceptive effects in mouse pain models (25–27, 29, 30).

P2Y,R have been found to be present in nociceptive pathways and have been associated with anti-nociceptive effects in several pain models (77, 78). A potent analgesic action of the selective P2Y1R agonist MRS2365 in neuropathic and acute pain has been recently reported (78). The coordinated regulation of GLYT1 and GLYT2 we report here is in good agreement with the anti-nociceptive properties of P2Y,R. Short term down-regulation of GLYT2 induced by P2Y1R stimulation might produce a transient increase in the levels of inhibitory synaptic glycine (61) that could be taken up by the activated GLYT1. Moreover, the stimulated GLYT1 may reduce glycine concentrations in the microenvironment of NMDA receptors, which would decrease excitatory neurotransmission (2). Hence, the resulting physiological consequence of the reported coordinated regulation of GLYT1 and GLYT2 by P2Y,R would be a net increase of the inhibitory pathways over the excitatory pathways, what may result in anti-nociception. The present regulation of glycine transport could be part of a homeostatic mechanism that might be deregulated in certain conditions as pain sensitization.

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REFERENCES
Regulation of GLYT5s by P2Y Receptors