The First Luminal Domain of Vesicular Monoamine Transporters Mediates G-protein-dependent Regulation of Transmitter Uptake*

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The activity of vesicular monoamine transporters (VMATs) is down-regulated by the G-protein α-subunits of Gαo2 and Gαq, but the signaling pathways are not known. We show here that no such regulation is observed when VMAT1 or VMAT2 are expressed in Chinese hamster ovary (CHO) cells. However, when the intracellular compartments of VMAT-expressing CHO cells are preloaded with different monoamines, transport becomes susceptible to G-protein-dependent regulation, with differences between the two transporter isoforms. Epinephrine induces G-protein-mediated inhibition of transmitter uptake in CHOVMAT1 cells but prevents inhibition induced by dopamine in CHOVMAT2 cells. Epinephrine also antagonizes G-protein-mediated inhibition of monoamine uptake by VMAT2 expressing platelets or synaptic vesicles. In CHOVMAT2 cells G-protein-mediated inhibition of monoamine uptake can be induced by 5-hydroxytryptamine (serotonin) 1B receptor agonists, whereas α1 receptor agonists modulate uptake into CHOVMAT1 cells. Accordingly, 5-hydroxytryptamine 1B receptor antagonists prevent G-protein-mediated inhibition of uptake in partially filled platelets and synaptic vesicles expressing VMAT2. CHO cells expressing VMAT mutants with a shortened first vesicular loop transport monoamines. However, no or a reduced G-protein regulation of uptake can be initiated. In conclusion, vesicular content is involved in the activation of vesicle associated G-proteins via a structure sensing the luminal monoamine content. The first luminal loop of VMATs may represent a G-protein-coupled receptor that adapts vesicular filling.

Communication between neurons in the central nervous system mainly occurs at specialized structures, the synapses. Variations in the input and output at synapses confer to synaptic plasticity, which involves changes at the post- and presynaptic sites, respectively. At the presynaptic site, availability and fusion competence of synaptic vesicles as well as the vesicular transmitter content contribute to the strength of postsynaptic answers.

Vesicular monoamine transporters (VMATs) translocate monoamines from the cytosol into the secretory vesicles of monoaminergic neurons, neuroendocrine cells, and platelets. Transport is driven by an electrochemical proton gradient (ΔμH+) across the vesicular membrane, which is generated by a vacuolar H+-ATPase (1). In mammals two closely related isoforms of the monoamine transporter, termed VMAT1 and VMAT2, respectively, were identified (2, 3). The transporter proteins presumably contain 12 transmembrane domains and are located on different vesicle subtypes (3–5). Both VMATs transport serotonin, dopamine, epinephrine, and norepinephrine but differ in their substrate preferences and affinities. In contrast to VMAT2, VMAT1 prefers epinephrine over norepinephrine, and the Kd for serotonin uptake is around 1 μM for VMAT1 but below 1 μM for VMAT2. Furthermore, histamine is only transported by VMAT2. The activity of both transporters is irreversibly inhibited by reserpine, whereas tetrabenazine exclusively inhibits VMAT2 (6, 7).

We have shown previously that monoamine uptake is regulated by heterotrimeric G-proteins that are associated with transmitter-containing secretory vesicles. The G-protein Gαo2 inhibits monoamine uptake in either VMAT1- or VMAT2-expressing neurons and neuroendocrine cells (5, 8, 9). A different G-protein, Gαq, blocks serotonin uptake in platelet granules that express VMAT2 (10). Interestingly, G-protein-mediated inhibition is lost in platelets derived from mice lacking peripheral TPH1 (tryptophan hydroxylase), which do not contain endogenous serotonin (11, 12). However, inhibition is restored when the secretory vesicles of TPH1-deficient platelets are preloaded with serotonin or norepinephrine (10, 13). These findings indicate that the monoamines stored inside the vesicle control the ability of G-proteins to regulate VMAT activity. However, it is not known how the intravesicular serotonin concentration is sensed and how the signal is transmitted to the G-protein.

The present study was undertaken to shed light on the upstream signaling events involved in the down-regulation of monoamine uptake by G-proteins. As an approach, we used

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2 The abbreviations used are: VMAT, vesicular monoamine transporter; CHO, Chinese hamster ovary; SHT1B, 5-hydroxytryptamine 1B; SLO, streptolysin O; PBS, phosphate buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; GMP-P(NH)P, guanosine 5’-(β,γ-imido) triphosphate.
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CHO cell lines transfected with VMAT1 or VMAT2. Such cells express endogenous Gα12 but are devoid of monoamine transporters or monoamine synthesizing and degrading enzymes, allowing for unperturbed external manipulation of monoamines. We found that, similar to our previous observations in platelets, G-protein-mediated regulation is dependent on the loading status of the vesicles, but with differences between the two transporter isoforms. Furthermore, deletion mutagenesis of the first intravescicular loop of VMAT1 and VMAT2 revealed that this domain is essential for the manifestation of G-protein-dependent transport regulation, suggesting that it is the transporter itself that operates as an upstream sensor in the signal transduction to heterotrimeric G-proteins.

EXPERIMENTAL PROCEDURES

Antibodies—A mouse monoclonal antibody against Gα12 (clone 101.4 and 101.1) (14) and rabbit polyclonal antibodies against VMAT1 and VMAT2 (5) were kindly provided by R. Jahn (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany) . Rabbit polyclonal antibodies against VMAT1 and VMAT2 were also purchased from Chemicon Int. and Synaptic Systems (Göttingen, Germany), respectively. Horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG were obtained from Amersham Biosciences. Alexa Red- or Oregon Green-labeled anti-rabbit or anti-mouse IgG were purchased from MoBiTec GmbH (Göttingen, Germany).

Transmitters and Receptor Ligands—5-Hydroxy-[3H]tryptamine trifluoroacetate (serotonin; specific activity, 4.33 TBq/mmol) was purchased from Amersham Biosciences. The monoamines epinephrine, norepinephrine, dopamine, serotonin, and histamine were obtained from Sigma. The 5HT1B receptor antagonists isamolten, GR55562, and SB216641; the 5HT1B receptor agonists anipirtoline, CP94253, and CGS12066B; the α1 receptor agonist cirazoline; and the α1 receptor antagonist prazosin were purchased from Tocris.

Toxins—Streptolysin O (SLO) from β-hemolytic streptococci was kindly supplied by U. Weller (Institut Ray-Rockefeller, Baden-Baden, Germany).

Mice—Wild-type and peripheral tryptophan hydroxylase knock-out (Tph1−/−) mice were bred as described (11).

Construction of Mutant VMAT DNA—Rat VMAT2 DNA (accession number M97381) was used to create the deletion mutants of the first luminal loop, one with a substitution of Pro42—Val130 corresponding to 89 amino acids by 5′-ATCGGA-TCCATGTCCAGGTGTGTTCG-3′; VMAT1-2, 5′-CTCGAACCCCCAGGCTTCATCG-3′; VMAT1-3, 5′-CTCGAACCCCCAGGCTTCATCG-3′; VMAT2-4, 5′-CTCGAACCCCCAGGCTTCATCG-3′; VMAT2-5, 5′-CTCGAACCCCCAGGCTTCATCG-3′; and VMAT2-6, 5′-CTCGAACCCCCAGGCTTCATCG-3′.

PCR was performed using Pfu polymerase, and the amplifications from VMAT2-1/VMAT2-2, VMAT2-1/VMAT2-3, VMAT2-1/VMAT2-4, VMAT2-6, and VMAT2-5/VMAT2-6 were individually cloned into PCR-script vector from Stratagene. Subsequently, the VMAT2-1/VMAT2-2 and VMAT1-1/VMAT1-2 were subjected to a restriction digestion using HindIII and EcoRI from the respective PCR-script clones and ligated to either VMAT2-4/VMAT2-6 or VMAT2-5/VMAT2-6 to generate VMAT2ΔQ61-T113 (short loop, i.e. variable region deleted) and VMAT2ΔP42-V130 (no loop, i.e. loop substituted by 5′ Gly) (see Fig. 6), respectively. VMAT2 cDNA and the loop deletion mutants were subcloned into pCDNA 3.0 into HindIII and XhoI sites.

VMAT1 DNA (accession number M97380) was used to create a deletion mutant of the variable region from His62—Val117 using the following oligonucleotides: VMAT1-1, 5′-ATCGGA-TCCATGTCCAGGTGTGTTCG-3′; VMAT1-2, 5′-CTCGAACCCCCAGGCTTCATCG-3′; VMAT1-3, 5′-CTCGAACCCCCAGGCTTCATCG-3′; VMAT1-4, 5′-CTCGAACCCCCAGGCTTCATCG-3′.

PCR was performed using Pfu polymerase, and the amplifications from VMAT1-1/VMAT1-2 and VMAT1-3/VMAT1-4 were subjected to a restriction digestion using BamHI and EcoRI for VMAT1-1/VMAT1-2 and EcoRI and XhoI for VMAT1-3/VMAT1-4 from the respective PCR-script clones and then ligated to generate VMAT1ΔH62—V117. VMAT1 cDNA and the loop deletion mutant were subcloned into pCDNA 3.0 into BamHI and XhoI sites.

CHO Cell Lines—CHO cells were transfected with wild-type VMAT-DNA using calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. The medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection. Briefly, plasmid was mixed with calcium-phosphate precipitation. The cells were grown in 35-mm dishes, the culture medium was changed 4 h prior to transfection to achieve exponential growth at the time of transfection.
VMAT2 Mediates G-protein Coupling

Preloading with

| none | epinephrine | norepinephrine | dopamine | serotonin |
|------|-------------|----------------|----------|-----------|

CHOVMAT 1

![Graph 1](image1)

CHOVMAT 2

![Graph 2](image2)
washed in PBS containing 1 mM EGTA, detached, and seeded on 100 mm dishes. In some experiments the Lipofectamine™ transfection reagent (Invitrogen) was used instead according to the manufacturer’s instructions.

To create stable cell lines, geneticin was added 48 h after the transfection procedure, and the medium was changed daily. The remaining cells were isolated by limiting dilution, and the clones were analyzed by immunoblot analysis, immunofluorescence microscopy, and serotonin uptake for VMAT expression and activity. The VMAT1-expressing CHO clone (CHO14.6) used in some of the experiments was kindly provided by B. Nürnberg (Heinrich-Heine-Universität, Düsseldorf, Germany).

CHO and CHOVMA1 cells were cultured at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. CHO cells expressing wild-type or mutant VMAT2 were cultured in the same medium supplemented with 400 μg/ml genetin.

**Immunoblot Analysis**—The cells were collected and centrifuged for 5 min at 200 × g. All of the following steps were carried out at 4 °C. After resuspension in 320 mM sucrose, 4 mM HEPES, pH 7.3, supplemented with protease-inhibitors (pepsatin, leupeptin, and 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), the cells were homogenized using a ball homogenizer (40 cycles; EMBL Germany). The homogenate was centrifuged for 10 min at 1000 × g, and the postnuclear supernatant was spun down for 30 min at 360,000 × g. The resulting pellet was resuspended in sucrose buffer, analyzed for protein content, and subjected to SDS-PAGE. After transfer to nitrocellulose, the proteins were analyzed using the indicated antibodies and the ECL detection system (Amerham Biosciences).

**Immunofluorescence Microscopy**—The cells were grown on glass coverslips, washed twice with PBS, and fixed in 4% formaldehyde in 0.1 x phosphate buffer, pH 7.4, for 45 min at room temperature. After three rinses with PBS they were incubated with blocking solution containing 5% normal goat serum and 2% bovine serum albumin dissolved in PBS supplemented with 0.1% Triton X-100 for 1 h at room temperature. Incubation with VMAT1, VMAT2, and Gαo₂ antibodies diluted in blocking solution was performed at 4 °C overnight. After two rinses with PBS the cells were incubated in the dark with fluorescence marker labeled secondary antibody diluted in 2% bovine serum albumin in PBS supplemented with 0.1% Triton X-100 for 1.5 h at room temperature. After two rinses with PBS and one rinse in water, the coverslips were mounted on glass slides for fluorescence microscopic analysis.

**Confocal Laser Scanning Microscopy**—The localization of VMAT1, VMAT2, and Gαo₂ was visualized using a Leica TCS confocal laser scanning microscope and a 40× oil immersion objective. Fluorescent dyes (goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 594, obtained from Molecular Probes, Eugene, OR) were excited at wavelengths of 488 and 543 nm, respectively. Fluorescent signals from the green and red channels were collected sequentially using two filters at 498–535 and 587–666 nm, respectively. The data from the two channels were merged into two-color images (resolution 1024 × 1024 pixels).

**Serotonin Uptake into CHOVMA1 Cells**—The cells were collected and centrifuged for 5 min at 200 × g. Incubation with SLO was performed in KG buffer containing 150 mM potassium glutamate, 20 mM PIPES, 4 mM EGTA, 1 mM MgCl₂, 1 mM dithiothreitol, adjusted to pH 7.0 with KOH as described elsewhere. Briefly, the cells were incubated with SLO (about 500 hemolytic units/3–5 × 10⁶ cells) for 10 min on ice. Under this condition SLO monomers bind to the cholesterol of the plasma membrane. After removal of unbound SLO, oligomerization and pore formation can be initiated by elevation of the temperature, restricting permeability to the plasma membrane (5, 15).

Preloading of intracellular compartments with monoamines or receptor ligands was started by adding 500 μL of KG buffer containing 1 mM ATP supplemented with 1 mM ascorbic acid and 30 μM, 100 μM, 300 μM, or 3 mM of either epinephrine, norepinephrine, serotonin, dopamine, or histamine or with 500 nM of the indicated receptor ligands. Incubation was performed at 37 °C for 15 min. Preloading solution was removed by centrifugation (2 min, 1090 × g, 4 °C). The cells were washed in KG buffer and divided into individual reaction cups. Uptake was started by resuspending cells in 50 μL of KG buffer containing 1 mM ATP supplemented with 1 mM ascorbic acid and 40 nM [³H]serotonin. Additives such as the nonhydrolyzable GTP analogue GMP-P(NH)P or reserpine were applied during this step. Incubation was performed for 10 min at 37 °C and stopped by adding 1 ml of ice-cold KG buffer followed by rapid centrifugation. The pellets were then lysed in 0.4% Triton X-100 to determine radioactivity by liquid scintillation counting and protein content using the bicinchoninic acid method.

**Serotonin Uptake into Blood Platelets**—Mouse or rat platelets were prepared as described elsewhere (10). The pellets plate-
lets were resuspended in Tyrode-HEPES buffer containing 134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, 1 mM MgCl₂, pH 7.4. Preincubation with receptor ligands or preloading of platelets was performed in Tyrode-HEPES buffer at 37 °C for 30 or 15 min, respectively, before platelets were subjected to the permeabilization and uptake procedure (10).

Preparation of Synaptic Vesicles—Synaptic vesicles were prepared from whole rat brain as described (14). The 350,000 × g pellet was resuspended in potassium glutamate buffer by passing it five times each through 23- and 27-gauge needles, respectively. Then synaptic vesicles were divided into individual reaction cups. Uptake was performed as described above but was stopped by the addition of 400 μL of ice-cold KG buffer followed by rapid centrifugation at 440,000 × g for 10 min at 4 °C. The pellets were analyzed for radioactivity and protein content.

Experimental Design—All of the experiments presented were repeated at least two or three times. Individual uptake experiments were performed in triplicate.

RESULTS

G-protein-mediated Inhibition of VMAT Activity Is Reconstituted in CHO Cells and Depends on Preloading of Internal Storage Organelles—For measuring serotonin uptake into VMAT-containing intracellular vesicles, we permeabilized CHO cells transfected either with VMAT1 (CHOVMAT1) or VMAT2 (CHOVMAT2) with SLO. SLO creates stable pores in the plasma membrane, which allow for direct access of metabolites to the cell interior while effectively equilibrating all metabolite and ion gradients between the cytoplasm and the incubation medium (5, 15).

Permeabilized CHOVMAT1 and CHOVMAT2 cells exhibited robust ATP-dependent serotonin uptake that was inhibited by reserpine. Some serotonin accumulation was also observed when nontransfected CHO cells were used, but this probably represents unspecific adsorption because it was not affected by reserpine (Fig. 1A). Similarly, no reserpine-sensitive transport was observed in either transfected or nontransfected cells when the permeabilization step was omitted (Fig. 1A). To exclude that the residual transport activity by nonpermeabilized cells is due to the presence of the plasma membrane transporter for serotonin, uptake was also monitored in the presence of the serotonin inhibitor fluoxetine. No change was observed (not shown).

Immunoblot and immunofluorescence microscopic analysis confirmed that transfection resulted in robust expression of VMAT1 and VMAT2 in the respective cell lines (Fig. 1, B and C). Furthermore, CHO cells contain endogenous Gsα as shown by a previously characterized monoclonal antibody specific for Gsα (Fig. 1B). Double immunolabeling experiments in either CHOVMAT1 or CHOVMAT2 cells revealed a broad co-localization between Gsα and the respective transporter on internal membranes (Fig. 1D). Finally we determined uptake kinetics at different serotonin concentrations into SLO permeabilized CHOVMAT1 or CHOVMAT2 cells (Fig. 1E). Collectively, these data show that both CHOVMAT1 and CHOVMAT2 cell lines display serotonin uptake with properties typical for VMATs in other systems and furthermore that VMATs are localized to internal membranes that also contain endogenous Goα2.

To investigate whether VMAT activity is regulated by G-proteins, we performed uptake experiments in the presence of the nonhydrolyzable GTP analogue GMP-P(NH)P, which is commonly used as an effective activator of trimeric GTPases. As shown in Fig. 2 (first columns) GMP-P(NH)P did not inhibit serotonin uptake by VMAT1 and VMAT2 expressing CHO cells. However, when permeabilized CHOVMAT1 and CHOVMAT2 cells were preloaded with increasing concentration of various monoamines (epinephrine, norepinephrine, dopamine, serotonin, and histamine), G-protein-mediated inhibition of serotonin uptake became apparent (Fig. 2). These findings resemble our previous observations of the serotonin-depleted platelets of Tph1−/− mice, in which G-protein-mediated inhibition of serotonin uptake was dependent on preloading with serotonin or norepinephrine (10). Monoamine concentrations required for effective preloading of CHOVMAT cells were somewhat higher than for preloading of platelets (10). The reasons for this difference are unclear, but it is conceivable that this is related to the need of the VMATs to fill endosomal compartments that are considerably larger than secretory vesicles.

Next, we investigated whether there are differences between the two transporters with respect to regulation by G-proteins. In CHOVMAT1 cells GMP-P(NH)P-induced inhibition of serotonin uptake was observed following preincubation with each of the monoamines (Fig. 2, upper panel). Preincubation with epinephrine and serotonin was more effective than with other monoamines, but inhibition was highly significant in all cases (Fig. 2, upper panel).

In CHOVMAT2 cells monoamine preloading of the internal compartments also resulted in down-regulation by GMP-P(NH)P of VMAT activity, but with interesting differences. Norepinephrine was most effective, whereas for histamine preloading and subsequent inhibition of transport was less pronounced and required higher histamine concentrations during preloading (26% inhibition at a preloading concentration of 3 mM; data not shown). Surprisingly, preloading with epinephrine (even when applied at a concentration of 3 mM) failed to induce G-protein-mediated inhibition of VMAT2 activity (Fig. 2, lower panel), although epinephrine is known to be transported by VMAT2 (6).

Epinephrine Antagonizes GMP-P(NH)P-induced Inhibition of Serotonin Uptake—To further investigate the unexpected failure of epinephrine preloading in eliciting transport inhibition, we first checked whether epinephrine is indeed a substrate for VMAT2 in CHOVMAT2 cells. Serotonin uptake was measured in permeabilized CHOVMAT2 cells in the presence of increasing concentrations of unlabeled epinephrine. As shown in Fig. 3A (left panel), epinephrine was effectively competing with serotonin for uptake. We next investigated which of the phenotypes (activated or nonactivated G-protein) prevails if the vesicles are preloaded with a mix of epinephrine and another monoamine. For this purpose CHOVMAT2 cells were preloaded with norepinephrine either alone or in combination with a 10-fold higher concentration of epinephrine and dopamine,
As expected, preloading with norepinephrine induced G-protein-mediated down-regulation of serotonin uptake by VMAT2. This inhibition was prevented when in addition epinephrine was added to the preloading solution. Inhibition, however, prevailed when a combination of norepinephrine and dopamine was used, showing that the block of G-protein signaling is not only specific for epinephrine but also that epinephrine is capable of effectively competing with the signaling of the other monoamines.

A similar competition was observed in normal serotonin-containing permeabilized platelets. Here, G-protein activation reduced serotonin uptake by VMAT2 by 40%. This reduction was prevented when platelets were preloaded with epinephrine, whereas preloading with dopamine rather enhanced the inhibitory effect of GMP-P(NH)P (Fig. 3B). A similar pattern was observed when small synaptic vesicles isolated from brain were used. Again, epinephrine preloading reduced GMP-P(NH)P-mediated inhibition of serotonin uptake, whereas dopamine preloading (if any) had the opposite effect (Fig. 3C). Together these data suggest that epinephrine prevents G-protein-mediated down-regulation of monoamine uptake, probably by acting by means of an intravesicular receptor site.

Monoamine Uptake Is Differentially Regulated by 5HT1B and α1 Receptor Agonists Depending on the VMAT Isoform—Together with our previously published data on the regulation of monoamine uptake in platelets (10), our findings indicate that G-proteins down-regulate transmitter uptake by VMATs and that this effect depends on the degree of vesicular filling. The question then arises regarding how the vesicular monoamine content is sensed and how the signal is transmitted to the G-proteins. The presumed intravesicular monoamine sensors are pharmacologically different for VMAT1 and VMAT2 because
VMAT2 Mediates G-protein Coupling

![Graph](image)

**FIGURE 4. Involvement of a 5HT1B receptor-like structure in G-protein-mediated inhibition of serotonin uptake by VMAT2.** A, serotonin uptake of permeabilized blood platelets of Tph1−/− mice in the absence or presence of GMP-P(NH)P was compared in control platelets and after preincubation with 50 μM serotonin or 500 nM of the indicated 5HT1B receptor agonists (left panel). In a similar experimental design Tph1−/− platelets were analyzed without or with preincubation with 50 μM serotonin, 50 μM serotonin plus 500 nM of the 5HT1B receptor antagonist isamoltan, or 500 nM of the 5HT1B receptor agonist CGS12066B (right panel). GMP-P(NH)P-mediated inhibition of serotonin uptake is induced by preincubating platelets with serotonin or with the 5HT1B receptor agonists anpirtoline and CGS12066B; CP94253 has no effect (left panel). Induction of G-protein-mediated inhibition after preloading with serotonin can be prevented by application of the 5HT1B receptor antagonist isamoltan (right panel). The inset in A shows the values for serotonin uptake underlying the blotted GMP-P(NH)P-mediated inhibition of the right diagram. B and C, serotonin uptake of permeabilized blood platelets of wild-type mice (B) and rats (C) in the absence or presence of GMP-P(NH)P was compared in control platelets and after preincubation with 500 nM of the indicated 5HT1B receptor antagonists. All of the 5HT1B receptor antagonists abolished inhibition of serotonin uptake by G-proteins. D, preincubation with 500 nM isamoltan abolished GMP-protein-mediated inhibition of serotonin uptake into rat synaptic vesicles. E, serotonin uptake into permeabilized BON cells in the absence or presence of GMP-P(NH)P was compared in control cells, after preincubation with 500 nM of the 5HT1B receptor antagonist CGS12066B and after preincubation with 500 nM of the 5HT1B receptor antagonist isamoltan. G-protein-mediated inhibition of serotonin uptake was not affected by CGS12066B but was slightly reduced by isamoltan in these preferentially VMAT1-expressing cells. Tph1 knockout was set at 100%; the percentage of the respective GMP-P(NH)P-mediated inhibition is shown. The values represent the means of three samples ± S.D. Each experiment was repeated twice. The stars denote statistical significance according to Student’s t test.
FIGURE 5. Regulation of VMAT1 activity by $\alpha_1$ receptor ligands in CHOVMAT1 and PC 12 cells. A, serotonin uptake of permeabilized CHOVMAT1 (left panel) or CHOVMAT2 (right panel) cells in the absence or presence of GMP-P(NH)P was analyzed following preincubation with 500 nM cirazoline ($\alpha_1$ receptor agonist) or with 500 nM anpirtoline (5HT1B receptor agonist). Cirazoline induces G-protein-mediated inhibition of serotonin uptake into CHOVMAT1 but not in CHOVMAT2 cells, whereas the opposite is true for anpirtoline. The insets in A show the values for serotonin uptake underlying the blotted GMP-P(NH)P-mediated inhibition. B, serotonin uptake into permeabilized PC12 cells in the absence or presence of GMP-P(NH)P was compared in control cells and after preincubation with 500 nM of each the $\alpha_1$ receptor antagonist prazosine or the 5HT1B receptor antagonist isamoltan. Only prazosine overcomes the G-protein-mediated down-regulation of serotonin uptake into these VMAT1-expressing cells. Uptake in absence of GMP-P(NH)P was set as 100%; the percentage of the respective GMP-P(NH)P-mediated inhibition is shown. The values represent the means of three samples ± S.D. Each experiment was repeated twice. The stars indicate statistical significance according to Student's t test.
VMAT2 Mediates G-protein Coupling

A

Vesicular lumen

CHO

CHOVMAT2 ΔQ61-T113

CHOVMAT2 ΔP42-V130

Cytoplasm

B

CHO

CHOVMAT2 ΔQ61-T113

CHOVMAT2 ΔP42-V130

C

[3H]serotonin uptake

pmol/mg protein/10 min

serotonin (µM)

CHOVMAT2 ΔQ61-T113

CHOVMAT2 ΔP42-V130

D

Preincubation with norepinephrine and anpirtoline

E

Preincubation with epinephrine and cirazoline

GMP-P(NH)p-mediated inhib. (%)

GMP-P(NH)p-mediated inhib. (%)

VMAT2

VMAT2 ΔQ61-T113

VMAT2 ΔP42-V130

VMAT2 ΔQ61-T113

VMAT2 ΔP42-V130

VMAT1

VMAT1 ΔA321-117

GMP-P(NH)p-mediated inhib. (%)

GMP-P(NH)p-mediated inhib. (%)

*
serotonin uptake in PC12 cells that only express VMAT1 (2). Here, only the α1 receptor antagonist prazosine, but not the 5HT1B receptor antagonist isamoltan prevented G-protein-mediated down-regulation of serotonin uptake (Fig. 5B).

Taken together, these data show that the pharmacological profile of G-protein activation differs between VMAT1- and VMAT2-expressing cells, even if the analysis was performed against the background of an otherwise identical CHO cell line. We therefore hypothesized that the luminal domains of the transporters themselves function as upstream sensors. This hypothesis was tested in the following experiments.

The First Luminal Loop of VMAT Mediates Down-regulation of Monoamine Uptake by G-proteins—To identify the putative receptor structure in the VMAT proteins, we first compared the sequence of the luminal domains of both transporter isoforms to identify regions in which the two transporters are different from each other. VMATs contain large intravesicular loops between transmembrane domains 1 and 2. The amino acid sequence diverges in the central part of the loop, particularly between amino acids Gln61–Thr113 (VMAT2) or amino acids His62–Val117 (VMAT1), whereas the rest of the predicted intravesicular sequences are almost identical (2, 3). Therefore, we created deletion mutants of both transporters in which either the divergent central part or the complete loop was deleted and generated stable CHO cell lines expressing these VMAT mutants. We then tested whether these transporters still show normal serotonin uptake, and if so, whether down-regulation of monoamine uptake by G-proteins is maintained.

For VMAT2, we created stable CHO cell lines expressing two mutants: VMAT2ΔP42–V130 (deletion of the entire loop) and VMAT2ΔQ61–T113 (deletion of the central portion of the loop; Fig. 6A). Expression of the mutant VMAT2 variants was verified by immunofluorescence microscopic analysis (Fig. 6B); no change of the intracellular distribution with respect to wild-type controls was observed. Uptake assays of permeabilized cells revealed that both mutated VMAT2 proteins transported monoamines in a reserpine-sensitive manner and with $K_m$ values that are indistinguishable from those observed for the wild-type protein (Fig. 6C). However, when we tested for G-protein-dependent regulation, we observed that neither in CHOVMAT2ΔQ61–T113 nor in CHOVMAT2ΔP42–V130 was serotonin uptake inhibited by GMP-P(NH)P following preloading with norepinephrine (Fig. 6D, left panel). A similar lack of signaling was observed when the 5HT1B receptor agonist anipirtoline was used instead during the preloading phase (Fig. 6D, right panel).

In a parallel approach, we also created a mutant form of VMAT1 in which the variable region of the first intravesicular loop was deleted, VMAT1ΔH62–V117. Again, G-protein-mediated inhibition of serotonin uptake induced by epinephrine or cirazoline was strongly reduced (Fig. 6E). We conclude that the first intravesicular loop of VMATs between transmembrane domains 1 and 2 represents a putative receptor-like structure, which senses the intravesicular monoamine concentration and signals to a trimeric G-protein to down-regulate transport activity.

**DISCUSSION**

Using CHO cells permanently expressing VMAT1 and VMAT2, we have shown that monoamines stored inside the vesicles represent the upstream signal that mediates inhibition of uptake by G-proteins. The vesicular monoamine sensors are encoded by the first intravesicular loop of the transporters that differ in the pharmacological properties between the two VMAT isoforms. Our data support the view that monoamine storage vesicles possess a feedback loop regulation that allows them to regulate uptake activity based on the degree of filling, and it is conceivable that this pathway is also used to control vesicular monoamine content by other signaling pathways. Remarkably, this regulation works for both VMAT1 and VMAT2, irrespective of whether the transporters reside on dense core (5, 8–10) or synaptic vesicles (10) or even on endosomal compartments in CHO cells (Ref. 28 and this paper).

The present work also has some more general implications. First, it reports on one of the rare examples for G-protein-mediated signal transduction occurring on endomembranes starting from the vesicular lumen. Second, G-protein-mediated down-regulation of monoamine transport is clearly not coupled to a heptahelial receptor. Third, to our knowledge it may be the first example of a G-protein-coupled receptor that is also a transmitter transporter.

Generally, VMAT activity depends on an electrochemical gradient, $\Delta\mu\text{H}^+$, driven by the vesicular proton ATPase. Independent from this basic regulation, variations in quantal size have been reported (16). Neurotransmitter content and consequently quantal size can be increased by overexpression of vesicular transporter molecules, as seen for the vesicular acetylcholine transporter (17), VMAT2 (18), and the vesicular glutamate transporter VGLUT1 (19). Accordingly, deletion of either VMAT2 (20–22) or VGLUT1 (19) reduces vesicular content. In addition, increasing the presynaptic γ-aminobutyric acid concentration (23) or the dopamine precursor DopAC

**FIGURE 6. Failure of G-protein-mediated inhibition of serotonin uptake in VMAT mutants lacking the first intravesicular loop.** A, schematic drawing of the VMAT2 deletion mutants used. The first two transmembrane domains forming the first intravesicular loop are shown. B, CHO cells, CHOVMAT2ΔQ61–T113 and CHOVMAT2ΔP42–V130 cells were subjected to immunofluorescence microscopic analysis using a rabbit antiserum against VMAT2. Both VMAT2 mutants are detected by the antibody when expressed in CHO cells. C, the kinetic analysis of CHO, CHOVMAT2ΔQ61–T113, and CHOVMAT2ΔP42–V130 expressing cells performed for 10 min at 37 °C reveals no difference from wild-type CHOVMAT2. The $K_m$ values are around 6–12 or 1–2 μM when analyzed either for 10 min at 37 °C or for 2 min at 29 °C (not shown), respectively. D and E, serotonin uptake into permeabilized CHO cells expressing wild-type or mutant VMAT2 (D) or wild-type or mutant VMAT1 (E) in the absence or presence of GMP-P(NH)P was compared after preincubation with 1 mM norepinephrine (D, left panel), 500 nM anipirtoline (D, right panel), 1 mM epinephrine (E, left panel), or 500 nM cirazoline (E, right panel). In contrast to CHO cells expressing wild-type VMAT2, epinephrine and anipirtoline failed to induce G-protein-mediated inhibition of VMAT2 activity. The inset in D shows as an example the values for serotonin uptake underlying the blotted GMP-P(NH)P-mediated inhibition following norepinephrine preloading. Deletion of the first intravesicular loop also declined G-protein-mediated inhibition of serotonin uptake by VMAT1 induced by epinephrine or cirazoline. Because of reduced expression transport activity of VMAT1 loop mutants, serotonin uptake was, however, only 25% of that for the wild-type VMAT1. Uptake in the absence of GMP-P(NH)P was set as 100%; the percentage of the respective GMP-P(NH)P-mediated inhibition is shown. The values represent the means of three samples ± S.D. Each experiment was repeated twice. The stars indicate statistical significance according to Student’s t test.
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(24) augments the amount of transmitter released. Interestingly, reducing the transmitter content does not change the overall vesicle morphology at least in mammalian neurons (25, 26). Collectively, these data indicate that secretory vesicles can vary their transmitter content depending on the physiological requirements. Because even in the central nervous system postsynaptic receptors are not saturated, a regulation by other means than the availability of transmitter and the electrochemical gradient is feasible (for review see Refs. 13, 27, and 37).

VMATs are not the only vesicular transporters regulated by heterotrimeric G-proteins. Functional heterotrimeric G-proteins have been found on glutamatergic vesicles (9), and the activity of VGLUTs is regulated by Go(2) (14).

All of the monoamines with the exception of epinephrine induce G-protein-mediated inhibition as expected for a general regulation of the VMATs. Epinephrine is transported by both isoforms but only effective in regulating uptake by VMAT1. In VMAT2-expressing systems, however, epinephrine overrides inhibition induced by other monoamines. Obviously features for transport and for inducing G-protein-mediated inhibition of transport or storage have to be distinguished. The abolishing effect of epinephrine is confirmed for all of the VMAT2-expressing systems investigated, although its physiological role is not clear so far. In addition VMAT1 and VMAT2 exhibit different receptor-like recognition domains identified by α1 or 5HT1B agonists and antagonists that appear to reside in the first luminal domain. So far a direct comparison of sequences from 5HT1B receptor and the first luminal loop of VMAT2 failed, however, to coincide.

Some more general considerations exclude the involvement of a classical heptahelical receptor. The relation between VMAT1 and α1 receptor-like structures and especially between VMAT2 and 5HT1B receptor-like structures applies to all of the systems (i.e. platelets regulated by Go(q) and synaptic vesicles regulated by Go(o2)). Assuming that a classical receptor initiates G-protein activation, this receptor should be the same for VMAT1 and VMAT2 at least in CHO cells. However, the differences between both VMAT isoforms are also maintained in the otherwise monoamine-free CHO system. Collectively these data strongly argue for the transporters themselves working as the upstream signal for G-protein activation. The first luminal domain of VMAT probably represents a receptor-like structure. Deletion of this loop may prevent the binding of the vesicular monoamines, thereby impeding the transduction over the membrane to the G-proteins localized at the cytoplasmic site of the vesicle.

So far a direct proof for vesicular monoamines as activators of vesicle-associated G-proteins, corresponding to the well known scenario of agonist-heptahelical receptor activation-G-protein activation, is lacking, and there is no easy way to obtain such evidence by direct experiments. It is also not clear whether the activated G-protein inhibits (directly or indirectly by using established down-stream signals) VMAT activity in a feedback reaction or whether it changes the storage capacity of secretory vesicles by other means. Cross-linking experiments or immunoprecipitation using antibodies against either Go(o2) or VMAT failed to show a direct interaction and were not successful in the past to confirm interactions between classical heptahelical receptors and their signaling G-proteins. Besides VMAT a putative candidate being addressed by the activated G-protein may be the calcium-dependent activator protein of secretion (CAPS1). Deletion of this protein leads to chromaffine granules with reduced catecholamine content, but the underlying molecular mechanism is unclear (29).

An epinephrine-sensitive, 5HT1B receptor-like structure may represent the putative upstream signal for G-protein activation by VMAT2-expressing vesicles irrespective of whether Go(o2) or Go(q) is the G-protein involved. Such promiscuity is also seen in the mouse β3b receptor, which utilizes both Go(q) and Go(o2) (30). It has to be pointed out that experiments were performed either in permeabilized systems (CHO, blood platelets) or on isolated SV. Even when permeabilized platelets activation of the thromboxane A2 receptor did not change vesicular serotonin uptake (10). In addition neither CHO cells (31) nor platelets (12) express 5HT1B receptors. Collectively, these data exclude the involvement of a classical heptahelical receptor of the plasma membrane. Our data may be best explained by assuming that the first luminal domain of VMATs mediates G-protein activation.

So far only one seven-membrane-spanning receptor resembling a classical G-protein coupled receptor has been found on endomembranes (32). The so-called KDEL receptor localizes to membranes of the Golgi apparatus and is involved in retrograde transport from the Golgi apparatus to the endoplasmic reticulum. Whether this receptor possesses the characteristics of a classical heptahelical receptor and which G-protein is involved remains unclear so far (33). Interestingly, Go(o2) proteins, which are the least understood with respect to upstream and downstream signals, appear to be preferentially not regulated by classical heptahelical receptors. For instance, the neuron-specific growth-associated protein GAP43 has been described to activate Go (34). Moreover, in Caenorhabditis elegans RGS7 (regulator of G-protein signaling 7) has Go(o2) inhibiting as well as promoting activity independent from a heptahelical receptor (35). The plasma membrane-associated amyloid precursor protein involved in Alzheimer disease also appears to be coupled to Go(o2) activation (36). Last but not least, Go(o2) regulates VMAT and VGLUT activity (5, 14, 37). In the present study we describe for the first time a signal transduction from the luminal site of secretory vesicles. Our data suggest that a transporter, such as VMAT, possesses in addition a receptor-like function localized in its first luminal loop that senses the intravesicular monoamine contents. In the case of VGLUT, the situation is probably more complex because the efficacy of G-protein regulation is linked to the cytoplasmic chloride concentration (14).

In summary the first luminal loop of VMAT probably exhibits receptor-like functions with pharmacological properties differing between the two isoforms. This loop is involved in the regulation of monoamine storage by heterotrimeric G-proteins. The cytoplasmic domains of VMAT mediating G-protein activation and the steps leading to reduced vesicular uptake probably involving cytoplasmic proteins have to be identified in the future.

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