Trafficking of the Plasma Membrane γ-Aminobutyric Acid Transporter GAT1

SIZE AND RATES OF AN ACUTELY RECYCLING POOL*

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Plasma membrane neurotransmitter transporters rapidly traffic to and from the cell surface in neurons. This trafficking may be important in regulating neuronal signaling. Such regulation will be subject to the number of trafficking transporters and their trafficking rates. In the present study, we define an acutely recycling pool of endogenous γ-aminobutyric acid transporters (GAT1) in cortical neurons that comprises approximately one-third of total cellular GAT1. Kinetic analysis of this pool estimates exocytosis and endocytosis time constants of 1.6 and 0.9 min, respectively, and thus approximately one-third of the recycling pool is plasma membrane resident in the basal state. Recent evidence shows that GAT1 substrates, second messengers, and interacting proteins regulate GAT1 trafficking. These triggers could act by altering trafficking rates or by changing the recycling pool size. In the present study we examine three GAT1 modulators. Calcium depletion decreases GAT1 surface expression by diminishing the recycling pool size. Sucrose increases GAT1 surface expression by blocking clathrin- and dynamin-dependent endocytosis, but it does not change the recycling pool size. Protein kinase C decreases surface GAT1 expression by increasing the endocytosis rate, but it does not change the exocytosis rate or the recycling pool size. Based upon estimates of GAT1 molecules in cortical boutons, the present data suggest that ~1000 transporters comprise the acutely recycling pool, of which 300 are on the surface in the basal state, and five transporters insert into the plasma membrane every second. This insertion could represent the fusion of one transporter-containing vesicle.

Plasma membrane neurotransmitter transporters, localized near neurotransmitter release sites, remove transmitter from the synaptic cleft and its vicinity (1). Impaired transporter function, and thus abnormal transmitter levels, is implicated in neurological disorders such as Alzheimer disease (2), epilepsy (3), Wilson disease (4), amyotrophic lateral sclerosis (5), and Parkinson disease (6). Several plasma membrane transporters are also targets of psychoactive stimulants such as cocaine and amphetamine. Due to their multiple physiological roles, plasma membrane neurotransmitter transporters are of extensive therapeutic interest.

The plasma membrane GABA1 transporter GAT1 is predominantly localized at the presynaptic terminal of GABAergic neurons and plays a major role in GABA uptake in the central nervous system. Data suggest that GAT1 can impact both tonic and phasic GABAergic transmission. For example, pharmacological blockade of GABA uptake through GAT1 prolongs the decay time of evoked GABA transmission (7). Also, in GAT1-deficient mice generated by homologous recombinant gene targeting, the extracellular GABA concentration is chronically elevated due to the lack of uptake; such elevation increases tonic GABA receptor conductance and decreases phasic GABA inhibition (8). These findings suggest that GAT1 regulates the time course of GABAergic transmission. However, the uptake process of the transporter takes ~100 ms, which is much longer than the time course of GABAergic transmission, suggesting that, at least in part, transporter action on neurotransmission is accomplished through binding and sequestration of released transmitter away from receptor sites (9). Thus, it is important to know the number of binding sites that transporters provide during neurotransmission and how that number might be regulated.

Previous studies show that GAT1 rapidly traffics between the plasma membrane and intracellular locations in neurons (10). Intracellular GAT1 resides on small synaptic-like vesicles that bear multiple intracellular trafficking molecules (10–12). In GAT1-expressing Chinese hamster ovary cells, the GAT1 internalization rate is ~10%/min at 22 °C (13). However, the number of acutely recycling endogenous transporters and the kinetics of this trafficking have yet to be determined.

The redistribution of transporters is subject to regulation. Multiple redistribution modulators have been identified, including second messengers (14–18), transporter agonists and antagonists (19–21), and interacting proteins (22–26). For example, the subcellular distribution of endogenous GAT1 in hippocampal neurons is under regulation by both protein kinase C and tyrosine kinase (13, 27, 28). Syntaxin 1A, a t-SNARE protein that interacts with GAT1, increases its plasma membrane expression (22, 29). Inhibiting tyrosine kinases pharmacologically or mutating tyrosine residues on GAT1 down-regulates GAT1 function by inducing an intracellular accumulation of the transporter that is correlated with an increased internalization rate (13). Previous data show more GAT1 intracellularly in the presence of PKC activators. However, it is not known whether such regulation occurs by chang-

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1 The abbreviations used are: GABA, γ-aminobutyric acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks’ balanced salt solution; TIR, transferrin receptor.
ing the number of recycling transporter molecules and/or rates of exocytosis or endocytosis. In the present experiments, modified biotinylation assays are applied to in vitro cortical neuron cultures to directly measure the basal trafficking rates of endogenous GAT1, to estimate the number of endogenous GAT1 molecules involved in acute trafficking, and to determine how three regulators of GAT1 trafficking exert their modulatory effects.

MATERIALS AND METHODS

Reagents—Immunoblotting reagents and [3H]GABA were obtained from Amersham Biosciences. Biotinylation reagents were obtained from Pierce. GAT1 antibody 342J was obtained from Dr. Nicholas Brecha (University of California, Los Angeles, CA). Protease inhibitor mixture tablets were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Neurobasal medium and B27 supplement were purchased from Invitrogen. All other reagents were obtained from Sigma-Aldrich.

Cell Culture—Primary cortical neuron cultures were prepared from E18 rat embryos. Briefly, the embryos were rapidly decapitated in accordance with protocols approved by the Animal Care and Use Committee of the University of Southern California. Cortices were dissected and placed in ice-cold Earle's balanced salt solution (in g/liter double distilled H2O: CaCl2, 0.294, MgCl2, 6H2O (0.1), KCl (0.373), and NaCl (9.35), pH 7.4). Cortices were cut into 2-mm pieces after careful removal of meninges. The small pieces were transferred to 5 ml of warmed Earle's balanced salt solution with 100 units of papain and incubated at 37 °C for 45 min. Tissue was triturated using warm culture Neurobasal medium supplemented with B27 supplement (0.5 ml t-glutamine). Neurons were plated onto poly-L-lysine-coated plates to a concentration of 106 cells/60-mm dish. Approximately 1 h after plating, neurons were observed to attach to the bottom of the plate, and then the medium was changed to warm fresh medium. Neurons were fed every 3–4 days by replacing half the medium with fresh medium.

CHO cell line was obtained from American Type Culture Collection and Minimum Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Cells were fed every 3 days.

[3H]GABA Uptake Assays—Cultured neurons were washed three times in HBSS and allowed to equilibrate for 10 min in the final wash. Inhibitors or activators were then applied to the incubation HBSS buffer for 10–30 min and removed before [3H]GABA was applied. The concentration of [3H]GABA was 30 nM, which was added to unlabeled GABA to obtain the desired final GABA concentration. The 15-min reaction was terminated by quickly washing the cells three times with HBSS. Cells were lysed in 0.05% SDS and divided in half. One half was used for measuring protein concentration, and the other half was used to determine amounts of [3H]GABA uptake.

Surface Biotinylation—Surface biotinylation experiments were performed as previously (13). To assess the surface expression level of GAT1, neurons were incubated in HBSS containing 1 mg/ml biotin at 37 °C. The remaining steps of the procedure were as described above. To calculate the kinetic parameters of GAT1 trafficking, total biotinylated transporter levels were measured (T). At each time point, the amount of transporter inserted into the plasma membrane, E(x)(t), was calculated as ΔT - T0 = T - T0, of which both T and T0 were measured directly. The time course of GAT1 exocytosis was well described by a single exponential, T - T0 = T0 e^(-kt).

RESULTS

The Acutely Recycling Pool of GAT1—Neurotransmitter-releasing vesicles can be categorized into readily releasable pools and reserve pools, based on their distinct trafficking kinetics. It is known that intracellular GAT1 molecules also reside on small synaptic-like vesicles. However, whether GAT1 trafficking has such heterogeneity or what might be the proportion of transporters involved in acute recycling is unknown. To estimate the acutely recycling pool size, we used modified biotinylation assays. First, to ensure that we could accurately assess the trafficking of GAT1, we verified the approach using the transferrin receptor (TfR). The subcellular trafficking of TfR has been extensively characterized using radioactive ligand binding (30), radioactive substrate accumulation (30), and monoclonal antibody binding (31). In these prior studies, 17–25% of total TfR was inserted into the surface of isolated fat cells during a 40-min incubation at 22 °C. We applied extracellular biotin to dissociated cortical neuron cultures under comparable conditions. The biotin would thus label all proteins present on the plasma membrane at the beginning of the assay, as well as those that had trafficked to the plasma membrane during the assay. Subsequently, biotinylated and unbiotinylated fractions were subjected to immunoblotting using anti-TfR antibody. Approximately 20% of total cellular TfR was found in the biotinylated (surface) fraction, comparable with previous estimates (Fig. 1A). This result suggested that biotinylation assays were providing an accurate assessment of the amount of protein resident upon and trafficking to the plasma membrane. Using this biotinylation approach, we then estimated the size of the acutely recycling pool for GAT1 when the neurons were in the basal state, compared with the total amount of cellular GAT1 (Fig. 1A). Extracellular biotin was incubated with cortical neurons at 37 °C for 30 min. To compare biotinylated GAT1 to total GAT1, 0.1 volume of whole cell lysate (T) and 0.5 volume of biotinylated fraction (B) were immunoblotted with anti-GAT1 antibody and quantified. Based upon 19 separate experiments, in the basal state, 31 ± 3% of total cellular GAT1 was found to visit the cell surface within a 30-min period. To confirm that we had reached a plateau in the time course of GAT1 recycling, we performed several experiments for 60 min.
Extending the recycling time did not reveal further increases in biotinylated GAT1, indicating that all the transporters that could be labeled in 60 min were already labeled within the first 30 min (Fig. 1B). These data show that, in the basal state, approximately two-thirds of total cellular GAT1 does not acutely traffic to the plasma membrane. However, it was possible that the biotin had reached its labeling capacity and thus failed to label all the recycling transporters. Three lines of evidence suggested that the result was not due to the saturation of biotin: first, higher concentrations of biotin (2 mg/ml) did not result in additional biotinylation (data not shown); second, all the transporters were able to be labeled when biotin was applied to permeabilized neurons; and third, parallel samples showed additional biotinylation of TfRs after 30 min (Fig. 1B). Thus we conclude that only one-third of total cellular GAT1 acutely traffics to the plasma membrane. We refer to this one-third as the “GAT1 acutely recycling pool.”

Exocytosis Rate of the Acutely Recycling Pool—The next question we addressed was how fast the GAT1 recycling pool traffics to the plasma membrane in the basal state. Because GAT1 accumulation on the plasma membrane saturated within 30 min, five points within that time frame (0, 2, 5, 15, and 30 min) were chosen to assess the GAT1 exocytosis rate (Fig. 2).

The biotinylated fraction at each time point represented the amount of GAT1 resident on the plasma membrane at the start of the assay and the amount that visited the plasma membrane during the assay time. A significant increase in biotinylated GAT1 was observed within 2 min. Within 15 min, the amount of biotinylated GAT1 reached a plateau. Using the equation described under “Materials and Methods,” the time constant τ was estimated to be 1.6 ± 0.4 min, thus yielding an exocytosis rate, rexo, of 0.7 ± 0.2 min⁻¹. These data suggest that on average, in the basal state, 22% of acutely recycling GAT1 molecules are inserted into the plasma membrane every minute.

Endocytosis Rate of the Acutely Recycling Pool—Direct estimation of endocytosis by biotinylation is problematic because some internalized protein is returning to the plasma membrane during the assay, and thus the endocytosis rate will be underestimated. To avoid such technical complications, we indirectly estimated endocytosis by measuring the combined effect of endocytosis and exocytosis, followed by subtraction of exocytosis. To do this, surface transporters were labeled by biotin and permitted to internalize for a given time (0, 2, 5, 15, and 30 min). The surface biotin was removed at the end of the time course by introducing a reducing agent extracellularly. The biotinylated GAT1 was then measured, which represents an intracellular accumulation of biotin-labeled GAT1 molecules. However, this measurement underestimates the true amount of endocytosis because a portion of the internalized biotin-GAT1 molecules subsequently returned to the plasma membrane and was subjected to the reducing agent. Thus, the amount of biotinylated GAT1 is the sum of true GAT1 endocytosis and subsequent GAT1 exocytosis. By measuring the rate of accumulation of biotinylated GAT1 and knowing the rate of GAT1 exocytosis, we solved for the rate of GAT1 endocytosis. The same approach has been used to estimate the endocytosis rate of synaptic vesicles (32).

As shown in Fig. 3, a significant accumulation was observed 5 min after the internalization assay commenced. A plateau was reached within 15 min, in which ~70% of surface GAT1 accumulated intracellularly; 30% of surface-labeled GAT1 thus represented transporters that did not internalize or were degraded or stripped of biotin after re-appearing on the plasma membrane. The data were fit by a single exponential function in which the rate constant rendo-exo was calculated as 0.25 ± 0.1 min⁻¹. We then calculated the endocytosis rate (rendo =
The Acutely Recycling Pool Size Is Regulated by Extracellular Ca\(^{2+}\), but Not by PKC Activators or Hypertonic Media—Multiple modulators regulate surface GAT1 expression. This could be due to either changing the size of the acutely recycling pool or altering the rates of exocytosis or endocytosis. We tested three known GAT1 trafficking modulators for their effect on the acutely recycling pool size: (i) PKC, a serine/threonine phosphorylation enzyme that decreases surface GAT1 expression (27); (ii) sucrose, which in part causes abnormal polymerization of clathrin and results in accumulation of GAT1 on the plasma membrane when applied to dissociated neuron cultures (10); and (iii) extracellular Ca\(^{2+}\), depletion of which results in reduced GAT1 surface expression (10).

To test the effect of PKC on the recycling pool size, 1 \(\mu\)M PMA was applied to dissociated cortical neurons for 10 min before and during biotin incubation (30 min at 37 °C). PMA decreased GAT1 surface expression by 2-fold as indicated by surface biotinylation assay (Fig. 4A); this replicated our previous findings (27). However, the pool size was not significantly changed compared with neurons without PMA treatment or compared with neurons treated with an inactive analogue of PMA, 4alpha-phorbol dibenoate (34 ± 4%, 35 ± 6%, and 35 ± 5%, respectively) (Fig. 4B). These data indicate that PMA decreases GAT1 surface expression without changing the recycling pool size.

We have shown previously that hypertonic medium containing sucrose inhibits internalization of GAT1 in a concentration-dependent manner (10). We applied sucrose to dissociated neurons at a saturating concentration (0.45 M) 5 min before and during biotin incubation (30 min at 37 °C). The treatment significantly increased \(^3\)H[GABA] uptake measured in parallel control cultures by 1.5-fold (Fig. 4C), which is consistent with our previous findings (10). As with PKC, the pool size measurement did not show significant differences between control neurons and neurons treated with 0.45 M sucrose (31 ± 5% and 32 ± 6%, respectively) (Fig. 4D). These data suggest that sucrose treatment can increase surface expression of GAT1 without changing the size of the GAT1 acutely recycling pool.

We and others previously reported that the recycling of internalized neurotransmitter transporters is dependent on extracellular Ca\(^{2+}\) (10); however, the mechanism underlying this effect is not yet clear. \(^3\)H[GABA] uptake in Ca\(^{2+}\)-free HBSS indicated a decrease in uptake to -60% (Fig. 4E). To test whether Ca\(^{2+}\) depletion had an effect on the recycling pool size, neurons were incubated in Ca\(^{2+}\)-free HBSS during the 30-min assay. As shown in Fig. 4F, biotinylated GAT1 was decreased by approximately one-half in Ca\(^{2+}\)-free HBSS when compared with the neurons in regular HBSS (1.2 mM Ca\(^{2+}\)). That is, the recycling pool size was decreased to 14 ± 3% of total cellular
GAT1. No further biotinylated GAT1 was detected by extending the incubation time up to 60 min (data not shown). To ensure that these calcium-dependent results were not due to nonspecific effects (e.g., on cell viability), we repeated these experiments using the transferrin receptor. TfR trafficking was unaffected by the absence of calcium (data not shown). Thus, we conclude that calcium exerts its effects on GAT1 trafficking at least in part by changing the recycling pool size.

PKC-mediated Decreases in GAT1 Surface Expression Are due to an Increased Endocytosis Rate—In dissociated cortical neuron cultures, PKC activation by PMA decreased GABA uptake, which was correlated to a decrease in GAT1 surface expression levels; however, PMA did not alter the GAT1 recycling pool size (see Fig. 4B). Thus, we examined GAT1 exocytosis and endocytosis rates in cultures treated with PMA. No significant effect of PKC activation was detected on the exocytosis kinetics of GAT1. However, the kinetics of intracellular accumulation of GAT1 were changed (Fig. 5). The peak accumulation of GAT1 was seen 2 min after cells were placed at a temperature permissive for internalization. After 2 min, the intracellular accumulation of GAT1 decreased dramatically, largely following the exocytosis kinetics of GAT1 in the basal state. These data suggested that the internalization rate of GAT1 was increased upon PKC activation, which was indicated by the much faster saturation time of accumulation (under 2 min, which is the minimum time point measured in the experiment). This increase in internalization was not seen in cultures treated with 4α-phorbol didecanoate (data not shown). Thus, the effect of PKC activation is to increase the GAT1 endocytosis rate with no significant effect on the exocytosis rate.

The PKC-mediated Decrease in GAT1 Is Dynamin-dependent—The finding that the endocytosis rate of GAT1 was increased upon PKC activation raised the question of whether PKC activates a new endocytosis pathway with a faster rate or whether the same “basal” endocytosis pathway is operating at an increased rate. Basal trafficking of GAT1 and several other neurotransmitter transporters occurs via a dynamin-dependent internalization pathway (10, 17, 33, 34). Does PKC-induced internalization of GAT1 also require these components?

To address this question, we blocked the dynamin-dependent endocytosis pathway by overexpressing a mutant form of dynamin (K44A). K44A dynamin is a dominant-negative form of dynamin that is defective in GTP binding and hydrolysis (35). Chinese hamster ovary cells were transiently transfected with GAT1 without K44A. Maximal uptake was increased compared with cells expressing only GAT1 (Fig. 6B). These two groups of cells (one group expressing GAT1 alone and the other expressing GAT1 and K44A dynamin) were subjected to PMA treatment. Cells expressing GAT1 without K44A showed a 40% reduction in uptake after PMA treatment, which is consistent with our previous findings. However, this PMA-induced reduction was abolished in cells expressing both GAT1 and K44A. Maximal uptake was increased compared with cells expressing only GAT1 (Fig. 6C). These data suggest that K44A abolishes internalization of GAT1 not only in the basal state but also in the PKC-induced internalization state as well. Therefore, PKC-induced internalization is likely through the same pathway involved in basal internalization.

DISCUSSION

The regulation of plasma membrane transporters influences synaptic transmission (9, 36, 37). To understand the extent of
this influence, it is essential to understand the mechanisms underlying subcellular transporter trafficking. We and others have previously isolated transporter-containing synaptic-like vesicles that are potential mediators of transporter trafficking in axon terminals (10), and we have identified many signaling molecules that regulate subcellular transporter redistribution (12, 14, 15, 22, 24, 28). In this report, by using a modified biotinylation assay to study GAT1 trafficking, (i) we defined an acutely recycling pool of GAT1 in cortical neurons that, in the basal state, comprises approximately one-third of total cellular GAT1; (ii) we measured the exocytosis rate \( r_{exo} = 0.7 \text{ min}^{-1} \) and the endocytosis rate \( r_{endo} = 1.1 \text{ min}^{-1} \) of acutely recycling GAT1 in the basal state; and (iii) we demonstrated that distinct transporter redistribution signals exert their effects by differentially regulating the recycling pool size or selectively uncoupling rates of exocytosis and endocytosis.

Transporters constitutively recycle between the cytosol and plasma membrane (10, 18). Our estimate of the acutely recycling GAT1 pool size is \( \sim 30\% \) in dissociated cortical neurons in the basal state. At present, what the remaining 70\% of total cellular GAT1 represents is unclear. One possibility is that it represents a pool of vesicles similar to the reserve pool of neurotransmitter-containing vesicles. At *Drosophila* neuro-muscular junctions, 14–19\% of vesicles are found in the readily releasable pools, whereas the remainder comprise the reserve pool (38). In mammalian dissociated hippocampal neurons, FM1-43 dye destaining indicates a readily releasable pool of 32\% (39). Another possibility is that the acutely recycling pool represents all the GAT1 molecules resident on transporter vesicles and that the remainder represents GAT1 localized to other intracellular organelles such as endosomes, endoplasmic reticulum, trans-Golgi-networks, lysosomes, and so forth. GLUT4, a glucose transporter in skeletal muscle and adipocytes, shows heterogeneity of transporter intracellular pools, including localization to sorting endosomes, storage endosomes, and exocytic vesicles. GLUT4 insertion into the plasma membrane upon a given signal represents transporters recruited from distinct endosomes and replenishment among the pools (40, 41).

Our kinetic studies of GAT1 suggest robust recycling of the transporter. In the basal state, 7\% of total cellular GAT1, which is equal to 23\% of the acutely recycling pool, accumulates on the plasma membrane every minute. The exocytosis rate and the endocytosis rate of acutely recycling GAT1 result in one-third of the acutely recycling pool being resident upon the plasma membrane in the basal state. Using a similar assay, basal \( \alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor endocytosis and reinsertion rates were estimated to be \( \sim 0.1 \text{ min}^{-1} \) in dissociated neuronal cultures (42). Different experimental approaches have reported exocytosis rate constants of endogenous transferrin receptor (43), T-cell receptor (44), and low density lipoprotein receptor (45) as 0.14, 0.012, and 0.16 min\(^{-1}\), respectively. According to these estimates, the GAT1 exocytosis rate is by far the fastest (6 times faster than \( \alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors, 4 times faster than TIR, 50 times faster than T-cell receptor, and 4 times faster than low density lipoprotein receptor). The fast recycling rate of GAT1 may not be surprising, given its localization to the highly specialized exocytic and endocytic compartments of axon terminals (46–49).

By definition, both changing the recycling pool size and uncoupling rates of exocytosis and endocytosis would regulate surface GAT1 expression. Do modulators of GAT surface expression differentially regulate these two processes? PKC activation and extracellular calcium depletion decrease surface GAT1 expression, whereas hypertonic sucrose treatment increases surface GAT1 expression (10, 27). Our present results show that PKC activation and sucrose administration did not change the pool size of GAT1 recycling, whereas extracellular calcium depletion dramatically diminished the recycling pool size.

Regulation of the pool size suggests a vesicle sorting mechanism. This has been a question of major interest in studies of neurotransmitter-containing vesicle trafficking in the axon terminal. High external Ca\(^{2+}\) concentrations favor sorting of vesicles to the readily releasable pool, whereas a lower Ca\(^{2+}\) concentration (e.g., 0.2 mM) favors sorting of vesicles to the reserve pool (38). In our Ca\(^{2+}\) depletion experiments, the recycling pool size measured by accumulated insertion of GAT1 into the plasma membrane was significantly reduced. Previously, we showed that extracellular calcium was a positive regulator of GAT1 surface expression during potassium-induced depolarization in hippocampal neurons (10). The present results show positive regulation by calcium even in the absence of experimentally induced depolarization. The most likely explanation for this result is that dissociated cortical neurons in culture have robust activities that can be blocked by TTX and enhanced by bicuculline (42). The present results do not exclude the possibility that the kinetics of recycling are also changed; however, this is difficult to measure because even with incubation times of 60 min, biotin-labeled transporter quantities are very low. It is interesting that Ca\(^{2+}\) depletion did not completely abolish recycling as compared with biotinylation labeling at 4 °C. This raises the possibility that a constitutive exocytosis mechanism might exist which is not Ca\(^{2+}\)-dependent.

Uncoupling rates of exocytosis and endocytosis accounts for the PKC-induced redistribution of GAT1. The exocytosis rate is not changed, whereas the apparent endocytosis rate is increased. Unlike in the basal state, there is a clear two-component aspect to endocytosis in the presence of the PKC activator PMA (see Fig. 5). One possibility is that the decrease after the peak is due to internalized GAT1 rapidly recycling to the plasma membrane, where the biotin is stripped. Thus, the accumulation of biotinylated GAT1 only appears to decrease. However, more interesting is the possibility that PMA internalizes GAT1 via two different pathways with different kinetics or that there are distinct surface populations of GAT1, one population that is PMA-sensitive, and one that is not. The endocytic mechanism that operates during PKC activation is dynamin-dependent, suggesting that the same mechanism is utilized for both basal and PKC-regulated GAT1 endocytosis. Tyrosine kinase activation (13) and extracellular GABA (19) also decrease the endocytosis rate of GAT1. The selective regulation of endocytosis might be a regulatory step shared by other transporter family members that are regulated by signaling pathways such as phosphatidylinositol 3-kinase and protein phosphatase 2A (50–53).

An average bouton of \( \sim 1.3 \mu m^3 \) in cortex contains 3000–4000 GAT1 molecules (54). Based upon this estimate, our data suggest the following: (i) 900-1200 molecules of GAT1 are acutely recycling on and off the plasma membrane in the basal state; (ii) 300–400 GAT1 molecules are found on the plasma membrane at any given time; and (iii) 3–5 GAT1 molecules are inserted into the plasma membrane every second. If transporter-containing vesicles are similar to neurotransmitter-containing vesicles in that on average only one vesicle is released per second in the resting state (55, 56), three to five transporters may represent the fusion of one transporter-containing vesicle. Given a trigger for transporter redistribution, for example, G-protein-coupled receptors that activate PKC (27), there is the potential for a net removal of hundreds of transporters on the time scale of a minute, and such redistribution could impact the time course of GABAergic signaling.
