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INVITED REVIEW

Vesicular glutamate transporters as anion channels?

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Abstract Since the discovery of a putative Cl^- channel on synaptic vesicle (SV) membranes, attempts to establish its molecular identity have proven surprisingly difficult. Recent evidence has emerged to support the idea that the vesicular glutamate transporter (VGLUT), whose main function is to concentrate the excitatory neurotransmitter glutamate into SVs, can also act as the Cl^- permeation pathway. Here, I summarize studies investigating the putative Cl^- channel on SVs and discuss the possible roles of VGLUT-mediated Cl^- transport on glutamate loading.

Keywords VGLUT · Glutamate · V-ATPase · Synaptic vesicle · Chloride channel

Abbreviations

NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
NFA	Niflumic acid
DIDS	4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid

Introduction

Synaptic vesicle (SVs) are the storage organelles for classical neurotransmitters at presynaptic terminals. Like other organelles, the lumen is maintained at an acidic pH relative to the cytoplasm by the activity of the vacuolar-type H^+ ATPase (V-ATPase) [9, 26]. The V-ATPase is an electrogenic H^+ pump, meaning that it does not efficiently pump H^+ ions unless a counterion is present to move across the SV membrane that would dissipate the voltage developed by the H^+ movement [27]. Physiologically, the main cytosolic anion, chloride (Cl^-), plays a key role in the regulation of the proton electrochemical gradient. Indeed, in the absence of Cl^-, the movement of only a few H^+ ions is sufficient to produce a large membrane potential ($\Delta\Psi$). Under normal circumstances, cytoplasmic Cl^- provides a shunting current for H^+ influx, facilitating the formation of ΔpH. As such, the balance of ΔpH and $\Delta\Psi$ across the SV membranes is influenced by the permeability of the SV membrane to Cl^-, as well as the Cl^- concentration gradient. This balance has important physiological consequences, namely that the uptake of neurotransmitters into SVs has been found to be strongly influenced by the proton electrochemical gradient [8]. Biochemical analysis has demonstrated that glutamate uptake predominantly depends on $\Delta\Psi$ whereas the uptake of cationic neurotransmitters, such as acetylcholine and monoamines, is mainly dependent on ΔpH. Uptake of zwitterionic neurotransmitters such as GABA and glycine shows dependence on both components.

The classical assay to measure acidification involves loading the weakly-basic fluorescent dye, acridine orange (AO), into SVs. In SVs isolated from mammalian brains, such experiments found that high Cl^- concentrations in the assay medium facilitated the formation of ΔpH [7, 25, 38]. Support for a Cl^- channel on the SV membrane was furthered when a Cl^- flux was observed in clathrin-coated SV fractions devoid of V-ATPase activity [45]. Despite the physiological importance for neurotransmitter loading, the protein(s) responsible for the Cl^- conductance in SVs has remained enigmatic. In this review, I focused on an emerging model in which the vesicular glutamate transporters (VGLUTs) [39] provide a mechanism for Cl^- movement, and discuss how this

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Cl^- conductance could potentially influence glutamate loading into SVs.

Properties of Cl^- movement monitored in isolated SVs

SVs can be purified from native mammalian brains using standard biochemistry-lab equipment, making them well suited for subsequent biochemical analysis [19, 28, 40]. One important discrepancy from their in vivo state is that the isolation procedure causes SVs to loose their luminal contents, including their neurotransmitter content [5]. Nevertheless, by using these SV membrane "ghosts", Cl^- dependent acidification has been monitored with AO. This membrane-permeable weak-base is trapped within acidic compartments, where its fluorescence is quenched in a concentration dependent manner [42]. AO quenching can be initiated with the addition of ATP; however, this was not apparent in the absence of Cl^-. The enhanced quenching of AO in the presence of external Cl^- was interpreted as an index of both Cl^- influx, as well as the concomitant H^+ influx via the V-ATPase. Typically, the time constant of AO quench at 32 °C was ~ 30 s [25, 38], slightly slower than acidification kinetics of SVs measured with a pH-sensitive fluorescent protein in cultured hippocampal neurons [9]; but also see earlier estimates which were much faster [2, 13].

Glutamate transport into vesicles is mediated by VGLUTs. In comparison to Cl^-, glutamate-induced AO quenching exhibited relatively slower kinetics, indicating that a distinct mechanism is responsible for its influx. In addition, while the degree of AO quench by external Cl^- exhibited a linear and almost non-saturable increase with concentration [16, 17], the AO quench associated with glutamate transport saturated at <5 mM [17, 25], which was in agreement with the affinity of VGLUT for glutamate [25, 29]. It should be noted that glutamate uptake, as well as glutamate-induced AO quenching, required the presence of external Cl^-, suggesting a critical involvement of Cl^- in glutamate transport by VGLUTs (see below).

Blockade of the V-ATPase reversed AO quenching, presumably as a result of H^+ efflux via the V-ATPase. Interestingly, the rate of AO fluorescence recovery, i.e., alkalization, differed depending on how SVs were acidified [17]. When SVs were acidified by Cl^-, the recovery following V-ATPase inhibition was much faster than those SVs acidified by glutamate. One interpretation is that H^+ efflux is inhibited differentially depending on anion concurrently being transported through their respective carrier. Alternatively, glutamate could be acting as a luminal buffer, potentially stabilizing ΔpH in SVs. In any case, the rapid efflux of Cl^- from SVs would make it difficult to track radioactive $^{36}Cl^-$ using the conventional flux assay.

Molecular identity of Cl^- permeation pathway in synaptic vesicles; lessons from knockout mice

Intracellular Cl^- channels belonging to the ClC family (ClC-3, -4, -5, -6 and -7; [21]) are frequently located on various intracellular organelles. Naturally, some of the ClC-type Cl^- channel isoforms became potential candidates for the Cl^--dependent acidification of SVs. Some of the ClC-type Cl^- channel family including ClC-5 and ClC-7 turned out to mediate $2Cl^-/1H^+$ exchange [20] in which Cl^- influx would facilitate exit of H^+, therefore resulting in "de-acidification". Yet, simulations of luminal pH either with a $2Cl^-/1H^+$ exchanger or a Cl^- channel predicted more efficient acidification by the exchanger [43]. To investigate whether members of the ClC-type Cl^- channel family contributed to the Cl^- flux, and associated H^+ influx, Jentsch and colleagues used the AO assay with the addition of ATP [37]. Given the strong expression of ClC-3 in the brain, its SV membrane localization and, more intriguingly, its neurological knockout phenotype (severe hippocampal degeneration), it became the obvious candidate. When crude SV membranes derived from ClC-3 KO mice were examined, it was evident that Cl^- dependent AO quenching was attenuated approximately by half in KO samples compared to wild type, suggesting that ClC-3 mediates the Cl^- conductance in a majority of SVs. The remaining activity could be explained by the fact that other ClC isoforms, including ClC-4 are expressed in the brain. However, among the SV proteins tested, the expression of VGLUT1 (also referred as BNP1 at that time; see next sentences) was significantly reduced in ClC-3 KO vesicles and, in addition, glutamate transport activity was reduced by a similar degree. It was also puzzling that despite the clear reduction in glutamate transport into isolated SV fractions from ClC-3 KO brains, the amplitude of miniature EPSCs (mEPSCs) recorded from hippocampal slices from ClC-3 KO mice was not altered. Intriguingly, significant increase in mEPSC amplitudes as well as evoked EPSC amplitudes was recently reported in cultured hippocampal neurons derived from ClC-3 KO mice [15], indicating the involvement of ClC-3 in the regulation of the driving force for glutamate uptake. The authors reasoned that the contradictory results between the two studies may be due to the preparations used where the acute brain slices and isolated SVs included unexpected effects from neurodegeneration. Nevertheless, suspicions that glutamate transport and Cl^- induced acidification were linked, were bolstered by Bellocchio et al., who, in an isolated light membrane fraction from PC12 cells, demonstrated that overexpression of VGLUT1 stimulated an intrinsic Cl^- induced AO quenching [3]. These results raised the compelling proposal that VGLUT1 itself has a Cl^- conductance. Indeed, the reduction in Cl^- induced acidification observed in ClC-3 KO samples could be alternatively explained by the reduction of VGLUT1 expression rather than the loss of ClC-3 expression. The hypothesis that VGLUTs exhibit the

Cl^- channel activity was not considered entirely eccentric, because anion channel activities in the plasma membrane glutamate transporter family (EAATs) and their bacterial ortholog Glt_{Ph} had already been demonstrated [10, 11, 35]. This implied that, although VGLUTs and EAATs are structurally unrelated and utilize different driving forces, they may share similar, if not identical, properties in anion conduction to support transport of the common substrate.

In order to clarify the contributions of ClC-3 and VGLUT1 on Cl^--induced acidification of SVs, Schenck et al. compared both ClC-3 and VGLUT1 KO mice before and after the onset of neurodegeneration [36]. The results were striking in that the VGLUT1-deficient SV fraction showed a strong reduction in AO quenching, supporting the contribution of VGLUT1 on Cl^- permeation [3]. In contrast, a reduction in Cl^- induced acidification was not observed from SVs prepared from young ClC-3 KO mice, where no visible neurodegeneration had occurred. Therefore, previous observations in adult ClC-3 KO samples could be explained either by the reduction of VGLUT1 protein level, or the reduction of VGLUT1-laden SVs, presumably due to the degeneration of brain regions in which VGLUT1 is highly expressed; i.e. hippocampus.

The Cl^- conductance reconstituted

Studies from KO mice have several intrinsic pitfalls. Firstly, the loss of even a single gene often leads to developmental alterations that could affect parameters of interest. In fact, the reduction in SV numbers at presynaptic terminals, and appearance of irregular-shaped tubular structures in the hippocampal region were detected in VGLUT1-KO brains, indicating a deficit in SV biogenesis [12]. It was uncertain whether these morphological changes were correlated with the reduction in AO quenching observed in VGLUT1-deficient vesicles. Secondly, due to the limited supply of pure SV fractions from KO mice, the crude vesicle fraction, lysate pellet 2 (LP2), was used for AO assays. Although the LP2 fraction has frequently been used as a "SV-enriched fraction", it also contains other membranes such as lysosomes, plasma membranes, and endosomes. These contaminants could potentially confound interpretations of the experimental results. Despite these concerns, in studies from proteoliposomes, the Cl^- induced AO quenching was recapitulated only when recombinant VGLUT1 protein was successfully co-reconstituted with a bacterial thermophilic F_0F_1-ATP synthase, suggesting that no other components were required for Cl^- induced acidification of SVs [36]. The AO quenching was VGLUT1-dependent as liposomes with only T- F_0F_1-ATP synthase failed to show Cl^- induced acidification. Furthermore, the extent of AO quenching was VGLUT1-dose dependent. Finally, the VGLUT1-dependent AO quenching was effectively blocked by the Cl^- channel blocker, DIDS ([16], our unpublished observations), similar to the Cl^- conductance observed in isolated SVs. These results were largely replicated in slightly different conditions by Preobraschenski et al. [33].

While the experiments from KO mice and proteoliposomes reasonably ruled out a contribution of proteins besides VGLUT on Cl^- permeation in SVs, direct evidence that Cl^- ions can pass through VGLUT is still lacking. For example, Moriyama and colleagues failed to detect Cl^- transport into VGLUT-containing liposomes using the radioactive $^{36}Cl^-$ flux assay when subjected to a valinomycin-induced K^+ diffusion potential, although it seemed that VGLUT-liposomes accumulated slightly more Cl^- than protein-free liposomes [22]. In addition, they failed to monitor Cl^- influx with a Cl^- sensitive fluorescent probe (SPQ). One interpretation is that membrane voltage does not trigger Cl^- transport; however, using an almost identical system, Preobraschenski et al. more recently demonstrated that valinomycin-liberated K^+ did in fact induce VGLUT1-dependent Cl^- efflux from Cl^- preloaded liposomes [33]. At present, key questions remain whether VGLUTs do actually exhibit Cl^- conductance, and, if so, what triggers the permeation? Does the Cl^- conductance in VGLUTs exhibit rectifying properties? In this case, does the topology of VGLUTs in reconstituted liposomes affect the results? Assay systems with higher precision and quantitative measurements for both Cl^- and the driving force will be required to clarify these issues.

Chloride conductance observed in other members of the SLC17/type I phosphate transporter family

VGLUTs belong to the SLC17/type I phosphate transporter family that also include the type I phosphate transporters (NPT1~4), lysosomal sialic acid transporter (sialin), and the vesicular nucleotide transporter (VNUT) [34]. Interestingly, two of the structurally related transporters, NaPi-1 (NPT1; SLC17A1) and NPT4 (SLC17A3), were shown to exhibit an anion conductance when expressed in *Xenopus* oocytes [4, 6, 24]. In both cases, electrophysiological recordings revealed a large leak conductance in the presence of external Cl^-. The conductance was permanently open and the reversal potentials of the currents were within the range of the equilibrium potential of Cl^- in oocytes (20~30 mV). Both Cl^- currents were modulated in the presence of substrates for the relevant transporter, indicating an allosteric regulation of the Cl^- currents by the respective substrates [24]. Interestingly, the Cl^- induced AO quenching mediated by VGLUT1 was inhibited by the addition of glutamate [3, 33]. These results raised a possibility that members of the SLC17 family share a common Cl^- permeation pathway although no structural evidence to support this notion has been provided.

One caveat of this assay system was that Xenopus oocytes contain an intrinsic outwardly rectifying current, whose properties are quite similar to those potentiated upon the overexpression of NPTs, including sensitivity to Cl^- channel

blockers, voltage dependency and ion selectivity. For instance, the NaPi-1-induced current was inhibited by NPPB and NFA, but not by DIDS [6], which was exactly the same property of the intrinsic Cl^- current [31] (note that the Cl^- conductance revealed by biochemical assays in SV membrane and VGLUT1-liposomes was effectively blocked by DIDS [16], our unpublished observations). Further, the NaPi-1-associated current exhibited anion preferences ($I^->Br^->Cl^-$) that were identical to the intrinsic current, and slightly different from those in SVs ($Cl^->Br^-$) [41]. Therefore, although some control experiments were performed in the described studies, the possibility that the overexpression of those transporters somehow stimulated one of the endogenous Cl^- channels expressed in Xenopus oocytes could not be fully excluded [31]. Nevertheless, future studies on VGLUTs using this assay system, in comparison to the SLC17 family members, may help to provide new insights into mechanistic features of the proposed Cl^- conductance in VGLUTs.

Physiological implications of the Cl^- conductance for glutamate transport into SVs

Given that endocytosis of SVs from the presynaptic plasma membrane likely engulfs some extracellular fluid, the Cl^- concentration in newly formed SVs should be higher than the cytosol. This concentration gradient will then start to dissipate when the V-ATPase begins pumping H^+ ions into SVs. H^+ influx occurs with a time constant of 15 s, resulting in the transport of approximately 1,200 H^+ ions until equilibrium is reached [9]. Interestingly, the kinetics of glutamate uptake into SV exhibits a similar time course as H^+ influx [18]. In such a situation, how would the Cl^- conductance support glutamate transport into SVs? Firstly, it is possible that the Cl^- conductance in VGLUTs confers a shunting current for H^+ influx [3, 33, 36]. Secondly, although it is believed that glutamate transport is predominantly driven by membrane potential [16, 22, 23, 25], there have been some indications that efficient transport also requires a pH gradient, or a slightly lower luminal pH [3, 38, 44]. Thus, Cl^- influx through VGLUTs could indirectly potentiate glutamate loading by promoting optimal luminal pH (Fig. 1a).

The scenario involving H^+-associated Cl^- influx, on the other hand, would face severe constraints, including osmotic imbalance and net charge movement. Since the majority of glutamate is negatively charged, even at acidic luminal pH, the influx of both glutamate and Cl^- would force efflux of anions or influx of cations during the transport cycle. The concomitant anion efflux must be favorable because influx of cations, if any, would massively increase the osmolality in the vesicle lumen. Therefore, efflux of Cl^- through VGLUT1 has been proposed to maintain the balance of both net charge movement and osmolality during the transport cycle (Fig. 1b). In fact, when VGLUT1-liposomes were co-reconstituted with the proton pump and preloaded with high concentrations of Cl^-, ATP-dependent glutamate transport was potentiated [33, 36] (but see [22]). Whether this potentiation was due to the Cl^- conductance of VGLUTs, or the proposed allosteric activation on VGLUTs from the luminal side, remains unknown.

The proposed Cl^- conductance in VGLUT1 does not necessarily rule out additional mechanisms of Cl^- transport. A Cl^-–activated glutamate uniport model, for example, involves Cl^- directly binding to VGLUT, converting it to an open form, and allowing passive glutamate influx, merely as a result of the membrane potential. Bioenergetical considerations have

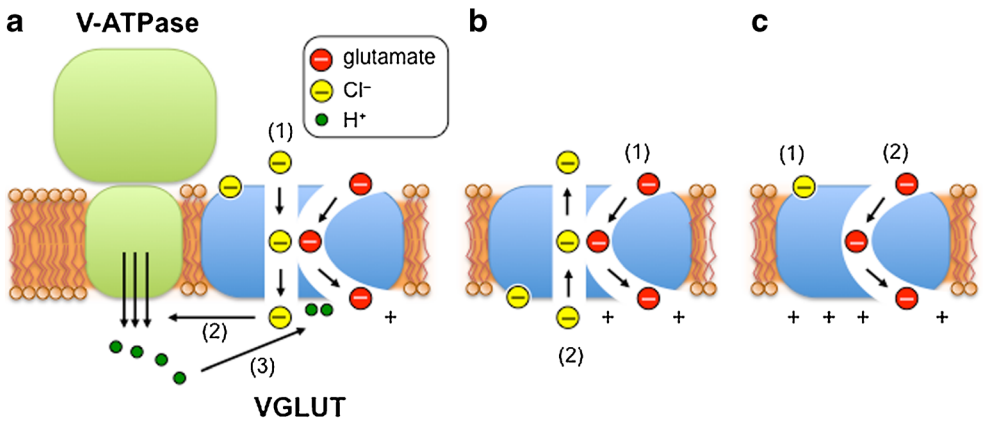

Fig. 1 Possible roles of Cl^- acting on VGLUTs during glutamate transport. **a** The Cl^- conductance in VGLUTs (*1*) indirectly facilitates proton pumping through the V-ATPase by supplying counterions for protons (*2*), thereby generating a larger ΔpH. In this model, it is predicted that protonation of VGLUTs at the luminal side is necessary for the activation of VGLUTs (*3*). Whether proton efflux is associated with glutamate transport is unknown. **b** Since glutamate is negatively charged within the range of physiological pH, the transport of glutamate would be more efficient when counterion movements take place (*1*). Following their endocytosis, SVs are likely to be filled with a Cl^-–rich solution. Therefore, Cl^- efflux through VGLUTs might help to maintain charge neutrality during glutamate transport (*2*). **c** Cl^- activation model. Cytoplasmic Cl^- allosterically binds and activates VGLUTs. In this model, glutamate transport is solely driven by the membrane potential, and predicts that other unidentified component in SVs might be responsible for the Cl^- conductance in SVs

suggested that a membrane potential 80 mV across the SV membrane would produce a glutamate concentration gradient of ~22. This would correspond to a glutamate concentration of up to ~100 mM if we assume a cytosolic glutamate concentration of ~5 mM [32].

Do other substrates pass through VGLUTs?

The Cl^- conductance in VGLUTs has been readily accepted, mainly because of the analogy with the plasma membrane glutamate transporters and other SLC17 family members. However, two VGLUT isoforms (VGLUT1 and 2) were originally identified as plasma membrane inorganic phosphate transporters, and in fact, the injection of both mRNA in Xenopus oocytes induced Pi uptake in a Na^+-dependent manner [1, 30]. Intriguingly, the Na^+-dependent ^{32}P uptake by recombinant VGLUT2 protein was recapitulated in proteoliposomes [23]. So, it is surprising that VGLUTs enable transport of two different substrates by using distinct driving forces. Moreover, the recent report by Preobraschenski et al. suggested that VGLUTs exhibited cation/H^+ exchange activity [33], which has been proposed in native SVs [14]. However, no evidence has been provided concerning K^+ movement through VGLUTs and, actually, a robust contribution of monovalent cations on the proton electrochemical gradient was not observed in pure SV fractions in previous studies [41]. Full biophysical descriptions on permeability of multiple substances through VGLUTs will require further prudent investigations.

Concluding remarks

Evidence has accumulated to suggest that VGLUTs serve as the Cl^- permeation pathway on glutamatergic SVs, and the Cl^- conductance may contribute to the control of the proton motive force, or, may be directly involved in glutamate transport by acting as a counterion. Recent studies have emerged that VGLUTs may transport other substrates, such as K^+ and inorganic phosphate. Whether VGLUTs indeed mediate so many transport activities with distinct driving forces is still an important question and will be the matter of investigation in the future. In addition, SVs storing other neurotransmitters such as GABA/glycine, monoamines and acetylcholine are also likely to require Cl^- channels for efficient vesicle acidification. The molecular identity of those Cl^- channels is, as yet, unknown.

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