Protein kinase C phosphorylates the system N glutamine transporter SN1 (Slc38a3) and regulates its membrane trafficking and degradation

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INTRODUCTION

Synaptic transmission at a chemical synapse is essential to many neuronal functions such as cognition, learning, and memory. It is based on exocytotic release of a neurotransmitter, its diffusion through the synaptic cleft and activation of specific receptors on the surface of the target cell. Glutamate is the major fast excitatory neurotransmitter in the central nervous system (CNS) undergirding the function of a wide range of synapses, while γ-aminobutyric acid (GABA) and glycine are the primary inhibitory neurotransmitters involved, among others, in synchronization of the principal neurons. In addition, monoamines, acetylcholine, neuropeptides, and other molecules sustain neuronal signaling at specific synapses [for review see Ref. (1)].

Sustained neurotransmission is dependent on replenishment of the neurotransmitters and efficient termination of the signal to reduce signal-to-noise ratio. In the case of the monoamines and acetylcholine, the neurotransmitters or choline (end-product of acetylcholine hydrolysis by acetylcholinesterase) are removed from the synaptic cleft by specific transporters on the nerve terminal membranes which also allow for their reuse in synaptic transmission (1). In contrast, the GABA transporter 3 (GAT3); and partially GAT1) and the major glutamate transporters (GLAST/EAAT1 and GLT-1/EAAT2) reside on surrounding astroglial cells, and a drain of these transmitters to the astroglial cells has been demonstrated (Figure 1) (2, 3). With the characterization of the members of the Slc38 family of amino acid transporters, showing that they may work in concert to shuttle glutamine from astroglial cells to neurons, the theory on a glutamate/GABA-glutamine cycle has been revitalized. In particular, the astroglial SN1, which releases glutamine, seems to be a major component of this cycle as it regulates extracellular concentrations of glutamine and shows dynamic membrane trafficking (4–6). In this review, we will discuss and consolidate recent data on the protein kinase C (PKC)-mediated regulation of SN1 and its functional implications.

THE GLUTAMATE/GABA-GLUTAMINE CYCLE AND ITS CONTRIBUTION TO THE REPLENISHMENT OF THE NEUROTRANSMITTERS GLUTAMATE AND GABA

The considerable amount of transmitter steadily released from neuronal synapses demands a dependable mechanism for replenishment. The neurotransmitters glutamate and GABA cannot be generated from the tricarboxylic acid (TCA) cycle intermediates because neurons lack the ability for anaplerosis due to lack of pyruvate carboxylase (7). Indeed, glucose alone is insufficient to sustain neurotransmission in brain slices (8). Shuttling of monocarboxylates and TCA cycle intermediates from astroglial cells to neurons and contribution to the formation of the neurotransmitters has also been suggested (7, 9), but it...
remains to be demonstrated that they can undergird neurotransmitter synthesis and synaptic transmission. The prevailing hypothesis is therefore that the fast neurotransmitters shuttle through perisynaptic astroglial cells in order to sustain neurotransmission. According to this glutamate/GABA-glutamine cycle and that astroglial-glutamine synthetase, which is enriched in astroglial cells and unique in being capable of synthesizing glutamine in the human body (13, 14). Glutamine transported into nerve terminals is catalyzed by the phosphatase-activated glutaminase (PAG), which is pronounced in nerve terminals, to resynthesize glutamate and GABA [for review see Ref. (15)]. Finally, the newly synthesized glutamate and GABA is accumulated inside synaptic vesicles by vesicular transporters prior to their exocytotic release (16, 17).

GABA as a precursor for the neurotransmitters glutamate and GABA has been demonstrated beyond any doubts (7, 8, 18–22). However, how glutamine is shuttled from astroglial cells, where it is synthesized, into neurons for its utilization has been enigmatic.

GLAST, GLT-1, and GAT3 and quickly remove the neurotransmitters from the synaptic cleft and away from their receptors, by binding the neurotransmitters and subsequently transporting them into astroglial cells [(10–12), review (3)]. The sequestered glutamate and GABA are readily metabolized to glutamine by glutamine synthetase, which is enriched in astroglial cells and unique in being capable of synthesizing glutamine in the human body (13, 14). Glutamine transported into nerve terminals is catalyzed by the phosphatase-activated glutaminase (PAG), which is pronounced in nerve terminals, to resynthesize glutamate and GABA [for review see Ref. (15)]. Finally, the newly synthesized glutamate and GABA is accumulated inside synaptic vesicles by vesicular transporters prior to their exocytotic release (16, 17).

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As the Slc38 family of amino acid transporters sustains astroglial-nuron transport of glutamine, regulation of the system A and system N transporters may have impact on neurotransmitter replenishment and synaptic plasticity. A better understanding of molecular mechanisms involved in their function and regulation may reveal novel (patho-)functional roles of these transporters and the glutamate/GABA-glutamine cycle and unveil novel therapeudic targets. Eukaryotic cells have an entire range of possible regulatory mechanisms to regulate the activity and expression of their proteins. In principle, all steps of production, maturation, trafficking, and degradation of a cellular protein can be regulated to control its expression levels, in addition to all types of direct or indirect influence of protein activity mediated by interaction with molecules ranging from protons to macromolecular protein complexes. The glutamine transporters are no exception to this rule. Classical biochemical experiments early demonstrated adaptive, hormonal, and osmotic regulation of system A and N activities as measured by functional transport assays (36–38). A nutrition hormonal, and osmotic regulation of system A and N activities (37, 42). Sophisticated regulation of SN1 activity in cell expressed, developmentally down-regulated 4-2) which adds on by releasing glycine for co-activation of NMDA receptors (35).

**A DIVERSE RANGE OF MECHANISMS ARE INVOLVED IN THE REGULATION OF THE SYSTEM A AND SYSTEM N TRANSPORTERS**

As the Slc38 family of amino acid transporters sustains astroglial-to-nuron transport of glutamine, regulation of the system A and system N transporters may have impact on neurotransmitter replenishment and synaptic plasticity. A better understanding of molecular mechanisms involved in their function and regulation may reveal novel (patho-)functional roles of these transporters and the glutamate/GABA-glutamine cycle and unveil novel therapeutic targets. Eukaryotic cells have an entire range of possible regulatory mechanisms to regulate the activity and expression of their proteins. In principle, all steps of production, maturation, trafficking, and degradation of a cellular protein can be regulated to control its expression levels, in addition to all types of direct or indirect influence of protein activity mediated by interaction with molecules ranging from protons to macromolecular protein complexes. The glutamine transporters are no exception to this rule. Classical biochemical experiments early demonstrated adaptive, hormonal, and osmotic regulation of system A and N activities as measured by functional transport assays (36–38). A nutrition signaling cascade that includes activation of phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) has been shown to be important for upregulation of system A (39, 40). For SAT2, an amino acid response element regulating the promoter activity has been demonstrated (41).

The system N activity and SN1 is regulated at the transcriptional and translational levels (37, 42). Sophisticated regulation of SN1 by interacting proteins and ions has also been demonstrated. SN1 is e.g., targeted by the ubiquitin ligase Nedd4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) which down-regulates SN1 activity in Xenopus laevis (X. laevis) oocytes (43). Insulin regulates expression of SN1 through the PI3K-mTor signaling cascade (44).

Protons regulate SN1 activity by competing with Na\(^+\) at the sodium binding site of SN1 as shown by increasing \(K_m\) for Li\(^+\) (a substitute for Na\(^+\)) with little change in \(V_{max}\) upon reducing extracellular pH (23). As Na\(^+\) binding to the Slc38 family is a prerequisite for the binding of the amino acid prior to its translocation, extracellular pH changes have profound effect on SN1 activity (23, 24). Protons also regulate SN1 at the mRNA level. SN1 expression at normal conditions is restricted to the S3 segment of the proximal tubules of the kidneys. During chronic metabolic acidosis (CMA), SN1 is induced also in the S1–S2 segments of renal epithelium, thereby increasing glutamine metabolism and generation of bicarbonate to counteract acidosis (6). Induction of CMA in rats results in upregulation of SN1 by about 10-fold at the mRNA level and more than 5-fold at the protein level (6, 42, 45). A pH responsive element in the 3' untranslated region (3'-UTR) of SN1 mRNA allows binding of specific proteins to the mRNA at low pH and thereby stabilizes the mRNA. As a result, SN1 expression is induced in the entire S1–S3 segments of the kidney (6).

**PKC-MEDIATED PHOSPHORYLATION IS CENTRAL FOR REGULATING MEMBRANE TRAFFICKING OF PLASMA MEMBRANE NEUROTRANSMITTER TRANSPORTERS**

For proteins transporting neuroactive compounds, such as dopamine, serotonin (SERT), GABA, and glutamate, membrane trafficking regulated by phosphorylation/dephosphorylation events has been shown as a common denominator. Nedd4-2/s serum and glucocorticoid inducible kinases 1 and 3 (SGK1 and 3) and protein kinase B (PKB) regulation have been described for the glutamate transporters EAAT1, 2, and 5 (46). However, the regulation by PKC stands out as a major mechanism. PKC isoforms and their numerous substrates regulate a variety of membrane proteins and in particular transporters [reviewed by (47, 48)]. In a comprehensive review, transporters for GABA (GAT1), SERT, dopamine (DAT1), and glutamate (EAAC1) were all shown to be regulated by PKC phosphorylation (48). For the three first transporters, PKC phosphorylation mediates internalization of the transporters, whereas dephosphorylation by Protein Phosphatase 2A (PP2A) or tyrosine phosphorylation mediates trafficking back into the cell membrane. For EAAC1, PKC seems to increase the surface expression together with PI3-kinase, whereas PP2A dephosphorylation elicits the internalization. Also the glycine transporter is being regulated by PKC (49–51). As PKC is ubiquitously expressed but strictly compartmentalized, its subcellular activation is differentially executed through a myriad of signal pathways, accounting for a large and diverse part of total phosphorylation phenomena in all cell types. Interestingly, PKC-signaling has been shown important for CNS processes like neuronal development, excitability, plasticity, and aging (52–54). Given the central regulatory role of PKC it is not surprising that the idea of PKC-regulation also of glutamine transporters was conceived by several different groups independently.

**EVIDENCE OF PKC-MEDIATED PHOSPHORYLATION OF SN1**

Protein kinase C has recently been shown to regulate SN1 under physiological conditions by two groups (Figure 1), however, there is a difference in the reported mechanisms involved (55, 56). Balkrishna and co-workers report that treatment of X. laevis oocytes expressing rat SN1 with the phorbol ester phorbol 12-myristate 13-acetate (PMA) results in a rapid down-regulation of glutamine uptake in <20 min. As this PMA-induced reduction in glutamine uptake is prevented by the specific PKC-inhibitor bisindolylmaleimide (Bis) and could not be induced by an inactive form of PMA (4a- PMA), the investigators conclude that PCK activation is involved. The specificity of the PKC action on SN1 was supported by lack of PMA-induced changes in the monocarboxylate transporter 1 (MCT1) activity in the same oocytes. The authors identify seven putative phosphorylation sites in the SN1 sequence, however, single or combined mutations of these sites have no impact on the PMA-induced down-regulation of SN1 activity. In an effort to further identify presence of particular regions or motifs on SN1 responsible for the observed PKC effect,
the SN1 cytosolic N-terminus was replaced with the N-terminal part of the homologous SAT1 protein. Treatment of the SAT1-SN1 hybrid with PMA still resulted in down-regulation of SN1, a result interpreted as proof that the targeting region is not localized in the SN1 N-terminus. Based on all these data it is concluded that SN1 is not directly phosphorylated by PKC; rather, the down-regulation of SN1 is mediated by some interaction with regulatory proteins endogenous to oocytes (55).

The authors also generate a fluorescently labeled construct of SN1, EGFP-SN1, and show that PKC-mediated down-regulation of glutamine uptake is caused by internalization of the fusion protein from the plasma membrane. The retrieval of SN1 from the plasma membrane is controlled by caveolin but remains dynamin-independent. Lastly, glutamine transport is challenged in the hepatocyte-derived HepG2 cells and cultured rat astrocytes; both hepatocytes and astrocytes have endogenous SN1 expression (26). PMA-treatment reduces glutamine uptake in cultured HepG2 cells but not in their cultured rat astrocytes, and this discrepancy is explained by differences in cell-specific regulatory mechanisms.

In the report by Nissen-Meyer and co-workers (56), we also detect a comparable time-dependent down-regulation of SN1 protein in the plasma membrane following PMA-treatment of mammalian cells stably transfected with SN1, and sequestration of the protein into intracellular reservoirs. The down-regulation is inhibited in the presence of Bis I, supporting mediation by PKC activation. However, we demonstrate PKC-mediated phosphorylation of SN1 in three different ways: first, direct phosphorylation of SN1 in vitro using a GST-fusion protein containing the N-terminal of SN1 and recombinant PKCα. Further, using site-directed mutagenesis to create unphosphorylatable SN1 mutants, we transfected cultured COS7 and PS120 cells with these mutant plasmids. Cells were then metabolically labeled with 32P-orthophosphate, stimulated with PMA and following immunoprecipitation and 2D-phosphopeptide mapping, we demonstrated that PKC-dependent phosphorylation in living cells was abolished selectively when a single serine residue was mutated (S52A) in the N-terminal of rat SN1, implicating this as the primary phosphorylation site.

Second, characterization of wild type and mutant SN1 in X. laevis oocytes electrophysiologically further corroborated our data on direct phosphorylation of SN1 by PKC. PMA-stimulation results in reduced SN1 activity as shown by abolished glutamine-induced inward currents. However, such reduction in the magnitude of the glutamine-induced inward currents perish when PKC is inhibited by Bis I. The unphosphorylatable S52A mutant resisted down-regulation in the presence of PMA, implicating that PKC isoforms phosphorylate SN1 at the S52.

Third, we also show direct phosphorylation of SN1 in cultured rat astroglial cells. By using specific affinity-purified antibodies selectively recognizing SN1 phosphorylated at the S52, we demonstrated that PKC stimulation results in increasing Bis I-sensitive phosphorylation of SN1 and that phosphorylated SN1 accumulates in intracellular compartments consistent with internalization of the protein. Such internalization of SN1 upon PKC-mediated phosphorylation is also supported by the fact that PKC activation significantly reduces \( V_{\text{max}} \) of the glutamine-induced currents in X. laevis oocytes but has no effect on the \( K_{\text{m}} \). Finally, our biochemical analyses suggest that SN1 may dynamically be recruited from these compartments upon dephosphorylation, however, prolonged activation of SN1 by PKC results in its degradation.

Interestingly, Sidoryk-Wegrzynowicz and co-workers recently also presented evidence that PKC is involved in the down-regulation of SN1 (57): Mn\(^{2+}\) exposure upregulates the activity of both PKCa and PKC\(\gamma\) in cultured astrocytes. Both enzymes were activated by phosphorylation and PKC\(\gamma\) was in addition activated by caspase 3-dependent proteolysis. In their experiments, PMA-stimulation for 4 h significantly down-regulates system N-mediated glutamine uptake in cultured astrocytes, an effect which was inhibited by addition of the PKC-inhibitor Bis II. In harmony with our work, they show that PMA reduces the SN1-content of biotinylated surface membranes long before 4 h. Although they did not succeed in co-immunoprecipitating SN1 with PKCa, they did show that SN1 co-immunoprecipitates together with PKC\(\gamma\) at 0 and 2 h, but not at the later times investigated. Thus, these experiments also lend support to PKC being an important regulator of SN1 protein cell surface expression under physiological conditions, albeit not demonstrating direct phosphorylation of SN1. Thus, there are compelling evidence and some indications that SN1 is directly phosphorylated by PKCa, PKC\(\gamma\), and PKC\(\delta\) and that this is followed by caveolin-dependent internalization of SN1.

**IS PKC INVOLVED IN THE DEGRADATION OF SN1?**

As shown above, PKC phosphorylates SN1 and regulates SN1 activity on cell membranes and thereby adjusts the transmembrane glutamine transport to comply with different (patho-)physiological demands for the neurotransmitter precursor. However, prolonged activation of PKC results in degradation of SN1 (Figure 1) (56, 57). SN1 interacts with Nedd4-2 when co-expressed in X. laevis oocytes and in astrocytes (43, 57). Moreover, stimulation of primary astrocytes with Mn\(^{2+}\) induces ubiquitin/proteasome-mediated degradation of SN1 via the Nedd4-2/SKG1 signaling pathway, thus providing a partial explanation for Mn\(^{2+}\)-induced neurotoxicity (58). Consequently, SN1-mediated transport increases when SN1 is co-expressed with the SKG1 and 3, and PKB (43). Thus, this pathway could represent a link to PKC-regulation since phosphorylation by PKC frequently is a way to tag proteins for ubiquitination and further lysosomal or proteasomal degradation (Figure 1) (59, 60).

**WHAT IS THE FUNCTIONAL SIGNIFICANCE OF SN1 REGULATION FOR THE GLUTAMATE/GABA-GLUTAMINE CYCLE?**

The glutamate transporters GLAST and GLT-1 and the GABA transporters GAT1 and GAT3 are enriched on the cell membranes of perisynaptic astroglial processes, capturing the exocytotically released neurotransmitters and translocating them into astroglial cells (10, 11). SN1 is also targeted to the same small glial processes (5, 26). Activation of the glutamate and GABA transporters will ensure that the local intracellular Na\(^{+}\)-concentration can be increased to a level where it can drive the SN1-mediated glutamine transport out of the cell (61), and as long as glutamine synthetase is present, glutamate and GABA imported will be
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PKC phosphorylates SN1

SN1 also sustains pivotal functions in peripheral organs. In the kidney, SN1 is localized on the basolateral membranes of the S3 segment of proximal tubules and is essential for glutamine metabolism. During CMA, K+–deprivation, and/or high protein intake, the total levels of SN1 increase significantly in the kidney and SN1 is also induced in the S1–S2 segments of the nephron (6, 45, 67). In the endocrine pancreas, we have shown complementary expression of SN1 and SAT2 and suggested that they work in concert to regulate a local glutamate-glutamine cycle and secretion of insulin (68, 69). The liver has one of the highest cellular concentrations of SN1 and is suggested to mediate glutamine influx for urea formation in periportal hepatocytes and glutamine efflux from the perivenous hepatocytes for transport of glutamine to other peripheral organs for cellular metabolism (26, 27). Accordingly, SN1 expression in the liver is regulated during starvation and insulin secretion (44). Thus, SN1 is essential in several physiological processes and may be differentially regulated in different organs to optimize a wide range of functions. As PKC isoforms are ubiquitously expressed throughout the body, but differentially in different cellular and subcellular compartments, isoform-specific PKC-mediated phosphorylation of SN1 may have a range of physiological and pathological roles.

CONCLUSION

Consolidated data from several papers on the regulation of SN1 (55–57) show that PMA-induced and Bsi I-inhibitable retrieval of SN1 occurs in several mammalian cell types, including primary rat astrocytes, and in X. laevis oocytes (Figure 1). Such membrane trafficking is governed by specific phosphorylation of SN1 at S52, selectively by PKCa and PKCy. Prolonged PMA-stimulation results in internalization by a caveolin-dependent and dynamin-independent mechanism and to degradation of SN1 through the Nedd4-2/ubiquitination pathway. PKC-mediated regulation of SN1 may, thus, be a key step in the regulation of the glutamate/GABA-glutamine cycle in the CNS and a wide range of pathophysiological processes in peripheral organs. Further studies are required for a better understanding of molecular mechanisms governing regulation of SN1 activity on the plasma membrane and its membrane trafficking and such studies may reveal novel mechanistic insight into a variety of physiological processes and to discovery of novel therapeutic targets.

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