Abolition of substrate-dependent currents by tyrosine mutation in the transmembrane domain of glutamate transporter

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Abstract By site-directed mutagenesis we examined the roles of tyrosine residues (Tyr^{127}) in the putative transmembrane domain of rat glutamate transporter (GLAST). When expressed in *Xenopus* oocytes, Y127F mutant protein, which was localized in plasma membranes of oocytes, completely abolished glutamate uptake currents but did not affect the intrinsic substrate-independent currents. Coexpression of wild type and mutant transporters supports that the Y127F mutation did not elicit glutamate efflux. The efflux of glutamate by wild type or Y127F mutant transporters was measured under the condition of ion perturbation where transporters run in the reverse direction.

Key words: Neurotransmitter transporter; Site-directed mutagenesis

1. Introduction

High-affinity glutamate transporters are known to modulate the amplitude and time course of synaptic transmission [1–3]. These transporters are also known to have pathological importance because altered levels of extracellular neurotransmitter concentration are potential causes of neurodegenerative disease. Cloning glutamate transporter cDNAs (GLT-1, GLAST, EAAC1, and EAAT4 [4–7]) facilitated understanding of the cellular localization of these transporters [8,9] and functional properties [10]. Site-directed mutagenesis study also revealed that polar or charged amino acid residues appear to be involved in glutamate or cosubstrate binding [11,12]. In this study, we examined three amino acid residues (Tyr^{127}, Phe^{253}, and Leu^{325}) located in the transmembrane domains of GLAST. These residues are conserved in all glutamate transporter isoforms, but substituted with other amino acid residues found at equivalent positions in the structurally related neutral amino acid transporter ASC1 [13] and SATT [14]. Tyr^{127} has an excellent nucleophilic hydroxyl group, Phe^{253} is conserved in the homologous region of the bacterial glutamate transporter GLTP [2,3], and Leu^{325} is replaced by a charged histidine in the corresponding site of ASC1. The functional impact of the mutant proteins was examined in the *Xenopus* oocyte system with a two electrode voltage clamp.

2. Materials and methods

2.1. Site-directed mutagenesis

GLAST cDNA was isolated from adult rat optic nerve mRNA by RT-PCR with primers, which was designed on the published GLAST sequence [5]. The forward primer is 5′-CAGTCACAGAATCAGAAAAGT-3′ (72–92); the reverse primer is 5′-ATCAGAGTAGGGAGGAAAGAG-3′ (1911–1931). Following reverse transcription, PCR was performed with denaturation at 95°C for 1 min, annealing and extension at 65°C for 1 min, and 7 min at 65°C for the last extension. The single 1.8 kb PCR product was cloned into the oocyte expression vector pGH19 [15]. We sequenced our PCR-derived optic nerve GLAST cDNA and found four differences in amino acids from the published sequences: from Phe to Leu at 50, from Asn to Lys at 275, from Leu to Val at 302, and from Thr to Ala at 432. These are located on the cytoplasmic or extracellular domain. Our GLAST nonetheless retains electrophysiological properties similar to those of the previous one. Site-directed mutagenesis was performed as described [16]. The mutagenic primers are GTGGTCTATTTCATGACTACC (Y127F), CTTCTTCATGTGCCTAGGCTTCGTGAT (F253L), and GTCATCGTCGGTCACCTCATTCATGCC (L325H). Mutation was done using the Trans Oligo *Aat*II/*Eco*RV selection primer (Clontech).

2.2. Electrophysiology

The defolliculated oocytes (stages V and VI) were injected with water or 10–20 ng of wild type or mutant cRNA. Oocytes were incubated in ND96 buffer, supplemented with 50 μg/ml gentamicin, 2.5 mM sodium pyruvate and 5% heat-inactivated horse serum [17]. Injected oocytes were maintained for 2–5 days at 18°C before use. Microelectrodes were filled with 1 M KCl and had a resistance of <2 ΩM. Steady state glutamate currents were measured by subtracting the currents in the presence of glutamate with the currents in the absence of glutamate. To monitor glutamate efflux, GLAST-expressing oocytes were tested under condition of ion perturbation where transporters run in the reverse direction, as described [18]. Briefly, oocytes were bathed in ND96 buffer and clamped at −50 mV. The medium was then replaced with a modified buffer (98 mM KCl, 0 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4) containing 10 mM L-glutamate. After currents were stabilized, L-glutamate was removed from the medium. 15 ng of wild type or Y127F cRNA alone or both were injected for experiments of glutamate efflux. Data are expressed as mean ± S.E.

2.3. Western blot

Oocyte plasma membranes were isolated as described [19]. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose, and pre-incubated (1 h) in blocking buffer (5% non-fat dried milk, 0.05% Tween 20 in 50 mM Tris, pH 7.4; 150 mM NaCl). Membranes were then incubated with an antibody specific to GLAST (0.6 μg/ml, provided by Jefferey Rothstein [20]). After several washes with TBS, blots were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000) for 1 h. Blots were washed and developed by chemiluminescence (Amersham).

2.4. Immunocytochemistry

Oocytes were fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 6 μm. Sections were treated with 3% hydrogen peroxide, 0.1% Triton X-100 in TBS for 20 min. Sections were pre-incubated (1 h) with 4% normal goat serum, 0.1% Triton X-100 in TBS, and then incubated for 48 h with an antibody to GLAST (0.6 μg/ml). After 1 h incubation with biotinylated anti-rabbit IgG (1:200, Vector Lab), sections were incubated (30 min) with avidin/peroxidase solution from the Vecstatin kit (1:100). The chromogenic reaction was performed with 0.5% diaminobenzidine, 0.1% hydrogen peroxide.

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3. Results and discussion

3.1. Expression of wild type and mutant transporters in Xenopus oocytes

Immunoblot of the oocyte plasma membranes shows that oocytes injected with wild type, Y127F or F253L cRNA expressed a relatively broad band with an apparent molecular mass of 60–70 kDa (Fig. 1). This heterogeneous band has been seen previously with antibodies to glutamate transporters [20,21]. Higher molecular weight bands probably represent aggregates of the transporters [22]. L325H cRNA-injected oocytes failed to reveal immunoreactive bands seen in wild type GLAST-injected oocytes. Water-injected control oocytes showed no immunoreactivity.

3.2. Electrophysiological properties of mutant transporters

Wild type GLAST in this study reveals similar electrophysiological properties of the previously cloned GLAST [23,24]. Application of L-Glu induced a steady state inward current (~100 nA at −90 mV) in oocytes injected with wild type GLAST cRNA (Fig. 2A). The inward current was also evoked by application of D-Asp, but not by the stereoisomer D-Glu (data not shown). Y127-injected oocytes (10–40 ng/oocyte) evoked no detectable currents ($n > 15$), whereas F253L-injected oocytes had a glutamate-induced inward current sim-

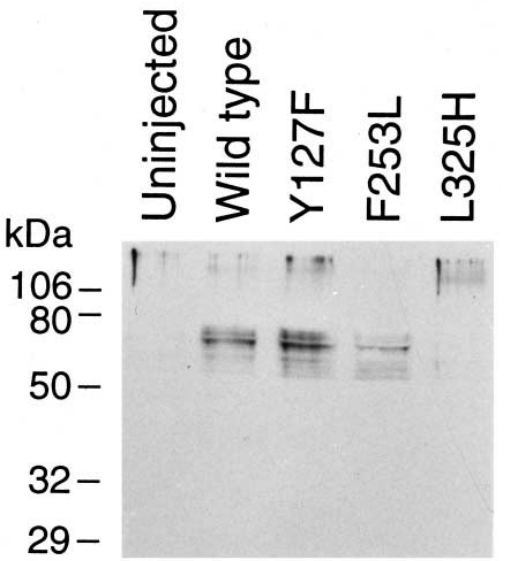

Fig. 1. Immunoblot of crude SDS extract of membranes prepared from *Xenopus* oocytes injected with wild type or mutant GLAST cRNAs. Proteins were immunoblotted with affinity-purified antibody to GLAST [20].

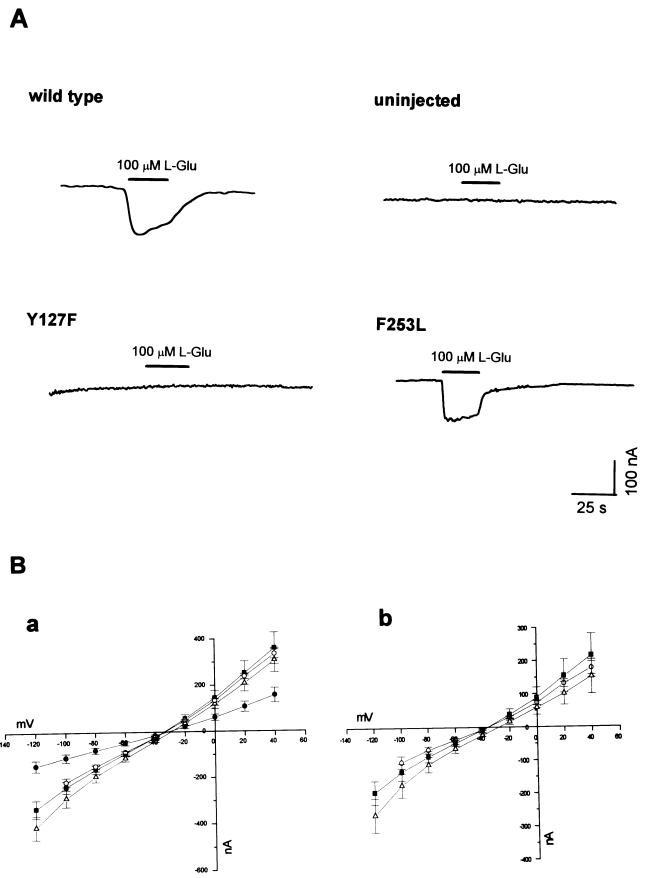

Fig. 2. A: The steady state inward currents evoked by application of 100 μM L-glutamate (holding potential: −90 mV). Bars: the duration of glutamate application. B: a: The current-voltage relation of uninjected (filled circles), wild type (filled squares), Y127F (open circles), or F253L (open triangles) cRNA-injected oocytes. The current was obtained in the absence of glutamate at various holding potentials. b: Voltage dependence of substrate-independent current between −100 and +40 mV. The substrate-independent current was acquired by subtracting the mean value of current in uninjected control oocytes from the current value in oocytes expressing wild type or mutant GLAST in the absence of glutamate.

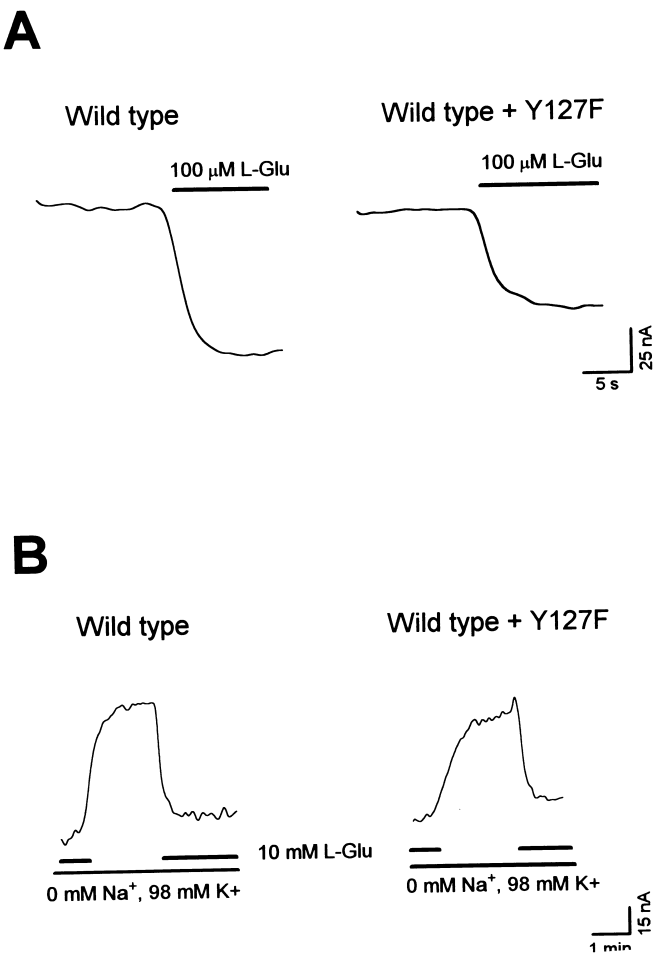

Fig. 3. Reverse transport of glutamate by wild type and Y127F mutant GLAST. A: An inward current in oocytes expressing wild type GLAST alone ($n=4$) or wild type plus Y127F mutant ($n=4$). The current was measured in the normal ND96 medium, with 100 μM L-glutamate at the −90 mV. B: Reverse transport of glutamate. Oocytes expressing wild type alone or wild type plus Y127F were placed in a buffer (98 mM K^+, 0 mM Na^+) containing 10 mM glutamate. The outward current was observed at −50 mV when glutamate was removed from the medium, as previously described [18].

ilar to wild type ($n=9$). Currents in Y127F mutants again failed to be induced with the application of D-Asp, or L-Ala, which is a substrate of neutral amino acid transporters (data not shown). However, mutations did not affect an intrinsic glutamate-independent ionic current of GLAST (Fig. 2B). This substrate-independent current, measured by subtracting currents of water-injected oocytes from those of GLAST-injected oocytes in the absence of glutamate [25], remained unchanged in oocytes expressing Y127F or F253L mutants. The apparent affinity for glutamate of F253L mutants showed no significant difference from wild type (data not shown). Oocytes injected with L325H mutant cRNA became unhealthy and died by 72 h after injection.

3.3. Glutamate efflux

Efflux of glutamate in oocytes expressing wild type or mutant GLAST was assayed by measuring the outward current in the presence of high [K^+] and low [Na^+] in the external medium [26]. For control experiments, oocytes were first put in the normal ND-96 medium (Fig. 3A). Application of 100 μM L-Glu, at which the currents are almost saturated (data not shown), evoked an inward current in oocytes expressing wild type alone or wild type plus Y127F mutant GLAST. The average amplitude of currents was 80.75 nA for wild type alone ($n=4$) and 74.75 nA for wild type and Y127F mutant ($n=4$) at a holding potential of −90 mV, showing no significant difference. Fig. 3B shows an outward current in wild type GLAST-expressing oocytes at −50 mV when glutamate was removed from a medium containing 98 mM K^+ and 0 mM Na^+. This indicates that the *glial* type transporter GLAST, similar to the neuronal transporter EAAC1 [18], could run in

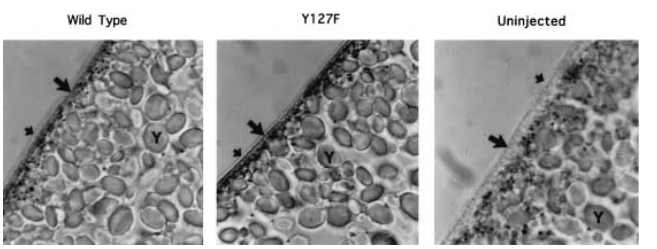

Fig. 4. Immunocytochemistry of oocytes expressing Y127F mutant GLAST. Sections of 6 μm were incubated with antibody against GLAST and stained using horseradish peroxidase-conjugated anti-rabbit immunoglobulin. Large arrows: plasma membranes with normal microvillus structure. Small arrows: vitelline envelope. Y: yolk platelets. *: the cortical granules.

the reverse direction in the presence of high extracellular Na^+ and K^+. Our results are similar to those seen with the glutamate transporter in retinal Müller glial cell [27,28]. Negligible currents were induced in oocytes injected with water or Y127F cRNA alone under the same experimental condition. The average amplitude of this outward current was 64 nA for wild type alone and 59.5 nA for coexpression of wild type and mutant. The ratio of the outward current to the inward current was therefore 0.79 for wild type alone and 0.78 for coexpression, suggesting no significant effect of mutant GLAST on the outward currents by wild type.

3.4. Localization of Y127F mutant transporter

Y127F mutant transporters were correctly targeted to the plasma membrane of oocytes (Fig. 4). An uninjected control oocyte was not immunoreactive. The dark-stained cortical pigment granules adjacent to the plasma membrane were not immunoreactive. The granule staining was strong in the animal pole and weak in the vegetal pole, and remained active even after treatment with hydrogen peroxide (3%) to quench peroxidase activity. Our immunocytochemistry result, in combination with the intact substrate-independent currents in Y127F-expressing oocytes, suggests that the absence of current is not due to a conformational change caused by the amino acid substitution. The exchange of tyrosine for phenylalanine is relatively conservative. They are aromatic amino acids with similar hydrophobicity but different hydrophilicity due to the hydroxyl group in tyrosine. Further, the membrane conductance of Y127F-expressing oocytes reveals no significant difference from the membrane conductance of wild type-expressing oocytes (Fig. 2B,a). Alternatively, we do not exclude the possibility that mutation at Tyr^{127} led to a 'non-conducting' transporter, without affecting glutamate binding. In this case, mutation might affect the 'turning' or 'operation' of the transporter rather than glutamate binding. A recent report by Stoffel's group shows that mutation of Tyr^{405} also abolished glutamate currents [11]. While Tyr^{405} resides in the intracellular domain, Tyr^{127} in this study is located in the transmembrane domain. Our immunoblot indicates that the oocytes injected with L325H mutant cRNA do not express transporter proteins. Instead, we observe high molecular weight bands, which appear to be aggregates of GLAST proteins. L325H protein failed to target to the plasma membrane (data not shown). We believe that this failure was caused by the conformational change deriving from a positively charged histidine. This substitution would create a different charge environment in the 'pore' region. Nonetheless, it is unclear how this cryptic activity in L325H mutants caused a low viability in oocytes. In conclusion, we demonstrate that mutation at Tyr^{127} abolished a glutamate-induced current. This residue appears to be critical for glutamate transport in glutamate transporters.

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