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A single residue contributes sensitivity to allosteric modulation of AMPA receptors by LY395153

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Abstract

Previous studies have shown that a single point mutation ($S^{750}Q$) in the splice variant region of rat Glu_1 subunits can eliminate positive allosteric modulation by cyclothiazide. The present study investigated the effects of mutating the equivalent residue ($S^{776}Q$) in the human Glu_4 subunit on the activity and binding of a novel AMPA receptor potentiator, LY395153 (*N*-2-(4-benzamidophenylpropyl-2-propanesulfonamide)). The mutation markedly attenuated, but did not eliminate, potentiation by LY395153 and cyclothiazide. In addition, binding of [^{3}H]LY395153 was significantly reduced by this mutation. These effects occurred in the absence of any change in the response to glutamate or the binding of a competitive AMPA receptor antagonist, [^{3}H]Ro 48-8587 ([2,4,5-^{3}H]9-imidazol-1-yl-8-nitro-2,3,5,6-tetrahydro[1,2,4]-triazolo[1,5-*c*]quinazoline-2,5-dione triethylammonium salt). Collectively, these results demonstrate that structurally diverse classes of potentiators are sensitive to mutations of this single Ser residue, suggesting that binding to this residue may be necessary for positive allosteric modulation of AMPA receptors.

Keywords: AMPA receptor; Biarylpropylsufonamide; Cyclothiazide

1. Introduction

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor family of glutamate receptors include four different genes termed Glu_{1-4} or Glu_{A-D}, and are expressed in nearly all of the central nervous system neurons. Alternative splicing of RNA for each subunit can give rise to flip (i) and flop (o) variants. These variants only differ by a 38-amino acid extracellular region positioned just before the transmembrane IV domain (Hollmann and Heinemann, 1994). AMPA receptor responses can be enhanced by positive allosteric modulators such as benzothiadiazines (e.g., cyclothiazide), which increase current flux through the ion channel in the presence of agonist (Yamada and Tang, 1993). A single residue within the flip/flop domain (Ser in flip and Asn in flop) has been shown to confer sensitivity to allosteric modulation by cyclothiazide in Glu_1 receptors such that when this residue is replaced by a Gln in the rat Glu_{1i} subunit, potentiation is eliminated (Partin et al., 1996).

LY395153 (*N*-2-(4-benzamidophenylpropyl-2-propanesulfonamide)) is a member of a structurally novel class of biarylpropylsufonamides that have been shown to positively modulate AMPA receptor activity (Baumbarger et al., 2001; Miu et al., 2001). Recent studies have shown that cyclothiazide can inhibit the binding of [^{3}H]LY395153 to native and recombinant AMPA receptors, suggesting that the Ser residue in the flip/flop domain also may be critical for modulation by LY395153 (Linden et al., 2001). This hypothesis was tested in the present experiments by investigating the effects of exchanging a Gln for the corresponding residue, Ser^{776}, in human Glu_{4i} receptors on the potentiation and binding of LY395153.

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2. Material and methods

2.1. Molecular biology

cDNA encoding human Glu_{4i} subunit subcloned into a pcDNA1 vector (Fletcher et al., 1995) was kindly provided by Dr. Keith R. Jarvie (Allelix Biopharmaceuticals, Mississauga, ON, Canada). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit protocol (Stratagene, La Jolla, CA). The complete coding region was then sequenced.

2.2. Expression of recombinant Glu_{4i} receptors in HEK293 cells

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose, L-glutamine, pyridoxine hydrochloride, and without sodium pyruvate and 10% fetal bovine serum in 5% CO_2 at 37 °C. Transfections were performed according to the FuGENE 6 (Roche Diagnostics, Indianapolis, IN) or LipofectAMINE 2000 (GIBCO BRL, Gaithersburg, MD) protocol. The pTracer-SV40 plasmid (Invitrogen, Carlsbad, CA) was co-transfected to identify successfully transfected cells and to visually estimate the percent of HEK cells transfected.

2.3. Binding studies

For the [^{3}H]LY395153 binding experiments, successful transfections were defined as greater than 75% GFP-positive cells after 48 h. Transfected cells were by resuspended in 10 volumes of ice-cold assay buffer (50 mM Tris–Cl, pH 7.5), disrupted with a Polytron homogenizer, and centrifuged at 20,000 × *g* for 20 min (4 °C). This procedure was repeated three times and then these pellets were stored at − 80 °C.

[^{3}H]LY395153 and [^{3}H]Ro 48-8587 ([2,4,5-^{3}H]9-imidazol-1-yl-8-nitro-2,3,5,6-tetrahydro[1,2,4]-triazolo[1,5-*c*]quinazoline-2,5-dione triethylammonium salt) binding was performed as previously described (Linden et al., 2001; Mutel et al., 1998). [^{3}H]Ro 48-8587 binding assays were initiated by addition of 100 μg of protein to an assay buffer (50 mM Tris–Cl, pH 7.5) containing different concentrations of [^{3}H]Ro 48-8587. Nonspecific binding was determined in the presence of 100 μM AMPA. Reactions were incubated for 2 h at 4 °C, rapidly filtered through GF/B filter and washed twice with 5 ml of ice-cold assay buffer. For [^{3}H]LY395153, binding was initiated by adding 30–50 μg of membrane protein to assay buffer (30 mM Tris–HCl, pH 7.5) containing 500 μM of L-glutamate and 40 nM of [^{3}H]LY395153. Nonspecific binding was determined in the presence of 6 μM cold LY395153. Reactions were incubated for 2 h at 4 °C in a final volume of 0.5 ml, rapidly filtered through GF/B filter presoaked in 0.3% polyethylenimine, and washed twice with 5 ml of ice-cold assay buffer. Radioactivity retained on the filter was counted in a scintillation counter (LS6500, Beckman-Coulter).

2.4. Electrophysiological recordings from HEK293 cells

Glutamate-evoked currents (holding potential − 80 mV) were recorded from isolated GFP-positive cells 30–54 h after transfection using conventional whole-cell voltage-clamp recording procedures (Baumbarger et al., 2001). Drugs were delivered using either a 16-barrel pipette array or a theta tube for concentration–response and fast perfusion studies, respectively.

2.5. Chemicals

L-Glutamic acid and (*S*)-AMPA were obtained from Tocris Cookson (Ballwin, MO). Cyclothiazide and LY395153 were synthesized at Eli Lilly and Company. [^{3}H]LY395153 and [^{3}H]Ro 48-8587 (specific activity 25 and 41 Ci/mmol, respectively) were obtained from Amersham (Piscataway, NJ).

3. Results

3.1. $S^{776}Q$ mutation of Glu_{4i} subunit reduces sensitivity to LY395153

Previous studies have shown that a single amino acid substitution within the flip/flop region from Ser to Gln ($S^{750}Q$) eliminates allosteric modulation by cyclothiazide in rat Glu_{1i} receptors (Partin et al., 1996). Given that cyclothiazide competitively inhibits the binding of [^{3}H]LY395153 to human Glu_{4i} receptors (Linden et al., 2001), the sensitivity of potentiation by LY395153 to mutation of the equivalent residue in human Glu_{4i} subunits ($S^{776}Q$) was tested. For wild-type receptors ($n = 14$), rapid application of glutamate (1 mM) produced an abrupt increase in inward current that rapidly desensitized (tau = 5.9 ± 0.3 ms; mean ± S.E.M) to a steady-state level that was 7.1 ± 1.0% of the peak response (Fig. 1A,E). In the presence of LY395153 (30 μM), the response to glutamate was markedly potentiated such that the magnitude of the current at the end of the 1.5-s pulse was 109.5 ± 3.5% ($n = 13$) of the peak response (Fig. 1A,E). For $S^{776}Q$ mutant receptors ($n = 12$), application of glutamate elicited currents that desensitized rapidly (tau = 5.3 ± 0.3 ms) to a low steady-state level (6.4 ± 0.9% of peak response) similar to wild-type receptors. However, the $S^{776}Q$ point mutation significantly reduced potentiation by LY395153 (30 μM) such that the current level at the end of the 1.5-s pulse was only 30.5 ± 2.6% ($n = 11$) of the peak response (Fig. 1C,E).

Concentration–response analyses indicated that the diminished activity of LY395153 was due, in part, to a decrease in potency at the $S^{776}Q$ mutant receptor. For wild-type receptors, application of LY395153 (0.03–10 μM) potentiated glutamate-evoked (100 μM, 10-s duration) responses in a concentration-dependent manner having an

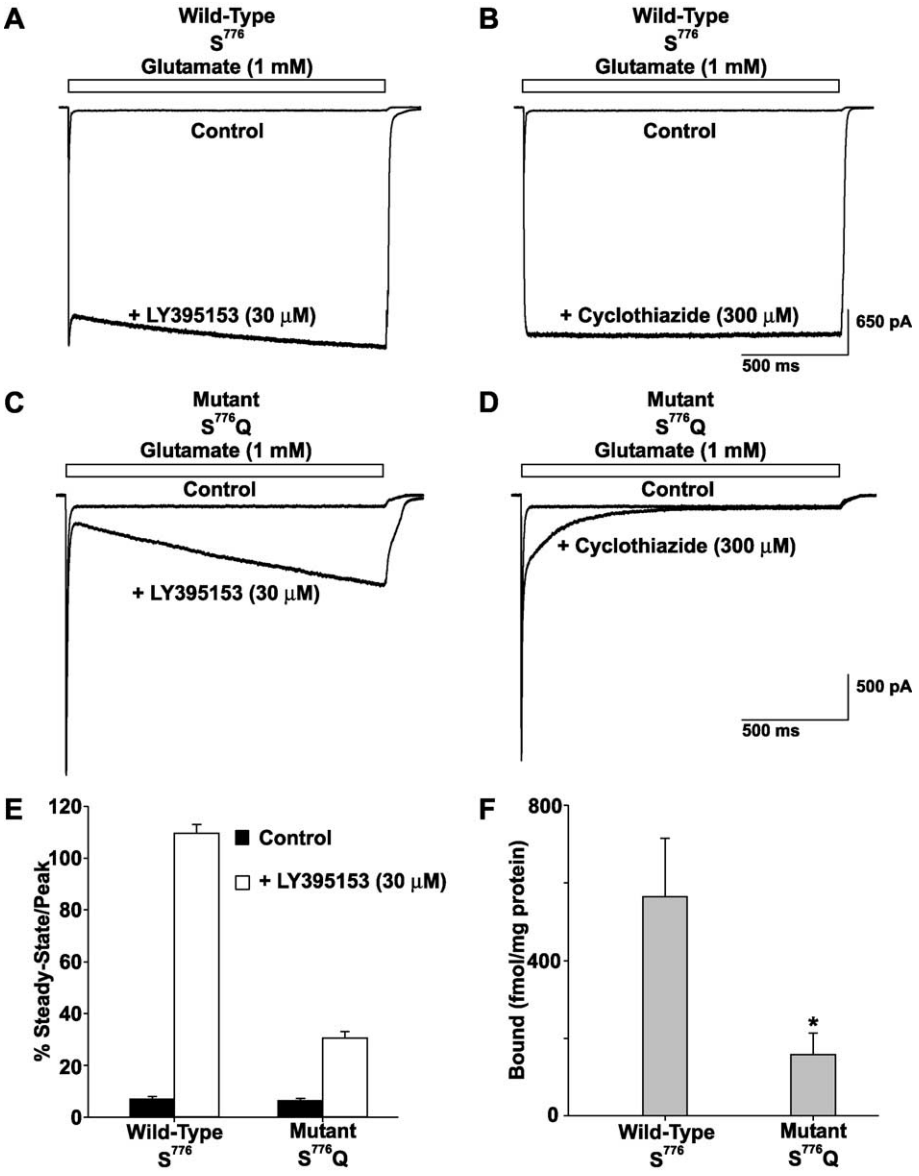

Fig. 1. The $S^{776}Q$ mutation significantly reduces sensitivity to LY395153 and cyclothiazide and binding of [^{3}H]LY395153 to Glu_{4i} receptors. (A–D) Responses of wild-type (S^{776}) (A–B) and mutant ($S^{776}Q$) receptors (C–D) receptors to 1.5 s rapid application of glutamate (1 mM) in the absence and presence of LY395153 (30 μM) and cyclothiazide (300 μM). (E) Histograms of the percent steady-state current to peak current of responses to glutamate alone (open bars) or when co-applied with of 30 μM LY395153 (solid bars) in wild-type and mutant receptors. Bars represent mean ± S.E.M. ($n = 11–14$). (F) Binding of [^{3}H]LY395153 to membranes from HEK293 cells transiently expressing wild-type or mutant receptors. Bars represent mean ± S.E.M. ($n = 4–5$; $P < 0.05$).

EC_{50} value of 2.6 ± 0.3 μM ($n = 6$; Fig. 2A,C). In comparison, the potency of LY395153 potentiation was markedly reduced by the $S^{776}Q$ mutation having an EC_{50} value of greater than 30 μM (Fig. 2B,C).

3.2. $S^{776}Q$ mutation of Glu_{4i} subunit reduces binding of [^{3}H]LY395153

The possibility that the decrease in potentiation by LY395153 at mutant receptors was due to a reduction in potentiator binding was investigated by measuring the binding of [^{3}H]LY395153 to recombinant wild-type and $S^{776}Q$ mutant receptors. Results showed that specific binding of [^{3}H]LY395153 (40 nM) was reduced approximately 70% by the mutation: wild-type = 564.8 ± 150.1 fmol/mg protein; $S^{776}Q$ mutant = 158.4 ± 54.9 fmol/mg protein (Fig. 1F). Although the marked reduction in [^{3}H]LY395153 binding precluded a saturation analysis, the result is consistent with the mutation significantly reducing binding of LY395153 to the receptor.

3.3. $S^{776}Q$ mutation of Glu_{4i} subunits does not alter response to glutamate

Previous studies have shown that binding of LY395153 to Glu_{4i} receptors is markedly enhanced in the presence of

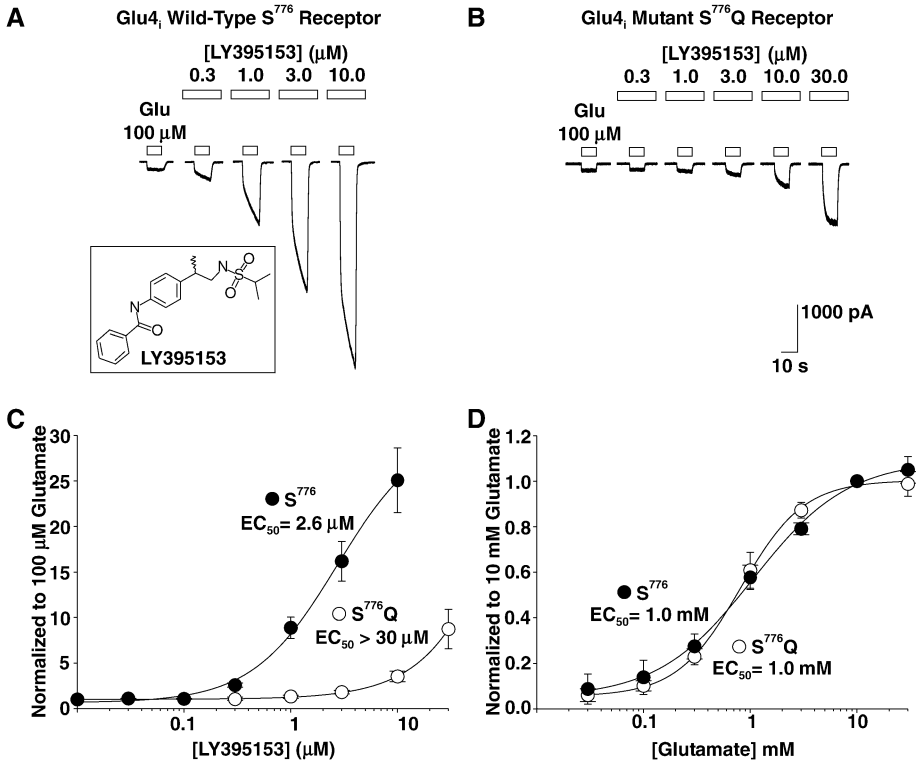

Fig. 2. Concentration–response profile for LY395153 and glutamate. (A–B) LY395153 potentiation of homomeric wild-type and mutant receptors was assessed by measuring the responses to 100 μM glutamate (Glu) alone and in the presence of potentiator (0.03–30.0 μM). Inset: Structure of LY395153. (C) Plot of the average degree of potentiation as a percent of the response to glutamate (100 μM) alone for each concentration tested. Points represent the mean ± S.E.M. (n = 4–6). (D) Plot of the average peak amplitudes of the current activated by each glutamate concentration (0.03–30.0 mM) normalized to the response to 10 mM glutamate. Points represent the mean ± S.E.M. (n = 3–7).

glutamate or AMPA (Linden et al., 2001). Conversely, the decrease in potency and binding of LY395153 in mutant receptors could be a consequence of a reduction in affinity and/or binding of glutamate to the mutant receptor. To investigate this possibility, the concentration–response relationship for glutamate was examined in both wild-type and the $S^{776}Q$ mutant receptors. The peak amplitude of the response to rapid application of glutamate (0.03–30.0 mM) was normalized to the maximal response (10 mM glutamate) and plotted as a function of glutamate concentration. The mutation had no effect on glutamate potency such that the EC_{50} values for wild-type (1.0 ± 0.2 mM, n = 6) and mutant (1.0 ± 0.2 mM, n = 6) receptors were not different (Fig. 2D). Consistent with these functional results, neither the affinity nor the maximal binding of the competitive antagonist, [^{3}H]Ro 48-8587 was affected by the mutation (wild-type K_D = 4.3 ± 1.0 nM, B_{max} = 1533 ± 890 fmol/mg; $S^{776}Q$ mutant K_D = 4.1 ± 1.8 nM, B_{max} = 1456 ± 335 fmol/mg). Thus, the effect of the mutation on potentiation by LY395153 does not appear to be due to a decrease in the binding and/or potency of glutamate.

3.4. $S^{776}Q$ mutation of Glu_{4i} subunits reduces sensitivity to cyclothiazide

Previous studies have shown that cyclothiazide can displace the binding of [^{3}H]LY395153 on wild-type human Glu_{4i} receptors (Linden et al., 2001). Therefore, the effects of cyclothiazide on the glutamate-evoked responses of wild-type and $S^{776}Q$ mutant receptors were examined. Cyclothiazide (300 μM) markedly increased the percent steady-state to peak current of glutamate (1 mM)-evoked responses in wild-type receptors to 98.2 ± 0.8% (n = 11) from 7.1 ± 1.0% (n = 14) in the absence of the potentiator. In contrast, cyclothiazide did not alter the percent steady-state to peak current amplitude in $S^{776}Q$ mutant receptors (control = 6.4 ± 0.9%, n = 12; cyclothiazide = 9.0 ± 2.0%, n = 9) (Fig. 1B,D). However, some minimal potentiation was detectable. Desensitization of the $S^{776}Q$ mutant receptor in the presence of cyclothiazide (300 μM) displayed a slow component and was best fit using a two-term exponential equation (tau_1 = 9.3 ± 1.2 ms, tau_2 = 135.5 ± 9.1 ms; n = 9). Similar results were found for a lower concentration (100 μM) of cyclothiazide (tau_1 = 7.7 ± 0.7 ms, tau_2 = 130.1 ± 9.0 ms; n = 5). Thus, while dramatically reduced, potentiation by cyclothiazide was still detectable in $S^{776}Q$ Glu_{4i} receptors.

4. Discussion

The present experiments demonstrate that mutation of the S^{776} residue to Q^{776} in the splice variant region of Glu_{4i} receptors significantly reduces potentiation of glutamate-evoked currents by LY395153. This effect is associated with

a marked decrease in the potency of LY395153 and a significant reduction in binding of [^{3}H]LY395153 to the receptor. These results suggest that the S^{776} residue is necessary for both binding of LY395153 to AMPA receptors and conferring the positive modulatory effects of the compound.

Previous studies have shown that the specific binding of [^{3}H]LY395153 can be enhanced in a concentration-dependent manner by AMPA receptor agonists (Linden et al., 2001). As such, the reduced potentiation and binding of LY395153 in the mutant receptor could be a secondary consequence of an alteration in binding of glutamate. Results suggested that this was not the case as neither the potency or desensitization kinetics of the response to glutamate nor the binding of [^{3}H]Ro 48-8587 differed from wild-type receptors. Thus, these results indicate that mutation of the S^{776} residue either (1) disrupts the binding site (directly or indirectly) for LY395153 or (2) alters the glutamate dependence of LY395153 binding.

The corresponding amino acids in Glu_{1i} and Glu_{1o} subunits are critical for the activity of the allosteric modulators, cyclothiazide, aniracetam, and thiocyanate (Partin et al., 1996). In addition, cyclothiazide and thiocyanate can displace specific binding of [^{3}H]LY395153 to membranes from rat cortex or HEK cells expressing wild-type Glu_{4i} receptors. However, the allosteric modulator CX516 does not displace [^{3}H]LY395153 binding (Linden et al., 2001). Collectively, these results suggest that many, but not all, of these structurally distinct compounds have overlapping binding sites that are sensitive to mutations of residue S^{776} in human Glu_{4i} subunits.

The minimal residual potentiation by cyclothiazide of Glu_{4i} receptor activity contrasts with the elimination of activity (at 100 μM cyclothiazide) by the equivalent mutation in rat Glu_{1i} and Glu_{1o} receptors. It is difficult to determine if this difference reflects an actual loss of a binding site in the rat Glu_{1i} and Glu_{1o} receptors, or if the affinity of cyclothiazide is sufficiently reduced so that the site is only functionally abolished. Nonetheless, this discrepancy suggests differences in the molecular determinants of allosteric modulation between subunits (Glu_1 vs. Glu_4) and/or species (human vs. rat). Consistent with this hypothesis, many potentiators exhibit differences in potency at homomeric Glu_{1-4} receptors (Miu et al., 2001). As the specific molecular characteristics of Glu_{1-4} subunits are elucidated, they may be exploited to develop compounds that potentiate a specific subset of native AMPA receptors.

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