The Lurcher Mutation of an α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor Subunit Enhances Potency of Glutamate and Converts an Antagonist to an Agonist*

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A point mutation of the GluR2 (A654T) glutamate receptor subunit converts it into a functional channel, and a spontaneous mutation at this site is thought to be responsible for the neurodegeneration of neurons in the Lurcher mouse. This mutation is located in a hydrophobic region of the M3 domain of this subunit, and this alanine is conserved throughout many of the glutamate receptors. We show here that site-directed mutagenesis of the homologous alanine (A636T; GluR1-Lc) in the GluR1 AMPA receptor subunit alters its channel properties. The apparent potencies of both kainate and glutamate were increased 85- and 2000-fold, respectively. Furthermore, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was converted from a competitive antagonist into a potent agonist. Our results demonstrate that a single amino acid within or near the putative second transmembrane region of the GluR1 subunit is critical for the binding/gating properties of this AMPA receptor.

Ionotropic postsynaptic glutamate receptors are responsible for most of the rapid excitatory synaptic transmission in the central nervous system. The binding of glutamate to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors generates excitatory synaptic currents in which the kinetics are determined by the relatively rapid gating of AMPA and the much slower gating of NMDA channels. Native channels consist of a heteromeric receptor composed of four subunits (1). A family of four different subunits (GluR1, -2, -3, and -4) can contribute to the formation of AMPA receptors, and each subunit can form functional homomeric channels (2, 3).

A related subunit, GluR2, with 25% homology to AMPA receptors, is found in cerebellar Purkinje cells; a spontaneous mutation of the GluR2 subunit is responsible for the neurodegenerative phenotype of the Lurcher mouse (4, 5). The GluR2 subunit cannot form functional heteromeric channels (6, 7), but spontaneously gated currents can be recorded in the absence of an agonist for the Lurcher mutation (GluR2-Lc) (5).

In this mutation, a substitution of a non-polar alanine with polar threonine occurs at position 654. This alanine is also conserved across a wide variety of glutamate receptors including the AMPA receptor subunits. It was postulated that neurons are lost in Lurcher mice as a consequence of spontaneous gating of GluR2-Lc channels and the resulting chronic depolarization (5).

The GluR2-Lc mutation is of particular interest because it demonstrates that an apparently nonfunctional channel can be converted to a functional one simply by a point mutation in or near the putative second transmembrane region. Mutational analysis of AMPA receptor gating has been targeted primarily to regions of the N terminus as well as the extracellular loop (8), but little attention has been paid to the possible role of the second transmembrane region in the gating of AMPA channels. To explore the possible role of this region in channel gating, we constructed a mutated GluR1 (flop) subunit with a threonine substituted for the alanine (A636T, GluR1-Lc) at the homologous position of the Lurcher mutation of the GluR2 subunit. We report here that the GluR1-Lc mutation has a profound effect on function of the GluR1 homomeric channel, converting an antagonist into an agonist and enhancing agonist potency.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—We introduced the Lurcher mutation into the analogous site of the AMPA receptor subunit GluR1 (GenBank™ accession number X7184, RNGLUR1) (9) by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) with the following primer: CAA CCT GGC TAC CTT CCT GAC (Fig. 1). The mutation converted alanine at position 636 to threonine. The mutated cDNA was subcloned into the pcDNA3 vector (Invitrogen Corp., Carlsbad, CA), and the open reading frame was sequenced in its entirety to ensure that only the Lurcher mutation was introduced. The cDNA was grown and purified with Qiaprep columns (Qiagen Inc., Mississauga, Ontario, Canada) and used for direct injection into oocytes or for in vitro translation.

Xenopus Oocyte Expression—The cDNAs for wild-type GluR1 (GluR1-wt) or GluR1-Lc, in the pCDNA3 vector were injected (10 ng) directly into the nucleus of stage V or VI Xenopus oocytes, or the cDNAs were in vitro transcribed with T7 RNA polymerase using an RNA transcription kit (Ambion) after linearization of the cDNAs with XhoI; 30 ng of RNA was then injected into the cytoplasm of the oocytes. Adult female Xenopus laevis were anesthetized with 0.15% aminobenzoic acid ethyl ester. The ovarian tissue was dissected and treated with 0.2% collagenase (Sigma) for 2 h in Ca2+-free solution and subsequently dechlorinated using fine forceps. Recordings were made from oocytes 3–6 days after the injection of cDNAs or cRNAs using conventional two-electrode, voltage clamp methods. The external bath was perfused with Ringer’s solution contained (in mM): 96 NaCl, 2 KCl, 1.8 Ca2+, 1.0 Mg2+, and 5 HEPES, pH 7.4. For EGTA-injected oocytes, 50 nl of 100
mM EGTA was injected into the cytoplasm 1 h before electrophysiological recordings. In some experiments, all extracellular Na\(^{+}\) and K\(^{+}\) was replaced with impermeant N-methyl-d-glucosamine (NMDG). All comparisons were made using Student’s paired \(t\) test or two-way analysis of variance.

Expression in HEK293 Cells—GluR1-wt and GluR1-Lc cDNAs (3 \(\mu\)g of cDNA/35-mm culture dish) were transfected using the Perfect Lipid Method (Invitrogen). Cells were plated to a density of about 10\(^6\) cells/35-mm dish, and after 24 h they were transfected with the appropriate cDNAs. Whole-cell patch clamp recordings were made 24–48 h after transfection using conventional techniques.

**RESULTS AND DISCUSSION**

Oocytes expressing the GluR1-wt subunit (\(n > 100\)) demonstrated an inward current (Fig. 2A, left), which decreased in amplitude as the oocytes recovered from insertion of the electrodes. Small inward currents were evoked from these oocytes in response to near saturating concentrations of glutamate (1 mM) relative to the agonist kainate (100 \(\mu\)M) (Fig. 2B) (\(n = 65\)). This difference in amplitude reflects the more limited desensitization of these receptors by kainate (10). The amplitudes of kainate-evoked currents remained constant over the period of recording. Oocytes expressing the GluR1-Lc subunit (\(n > 100\)) demonstrated larger initial inward currents that slowly inactivated over a period of 15 to 60 min (Fig. 2A, right). Furthermore, the superimposed peak responses evoked by glutamate or kainate slowly declined in parallel with the decay of this inward current (not shown). Each response to agonist also desensitized during the application of agonist (Fig. 2, B and C) (\(n = 75\)). This agonist-induced decay of currents exceeded that attributable to the spontaneous decay of the initial inward currents. Indeed, the cessation of each application of agonist was associated with an acceleration of inactivation that was manifest as a net outward current (Fig. 2C, and see below). The relative responsiveness to glutamate and kainate was also altered (Fig. 2C).

Qualitatively, responses to glutamate were nearly as large as those to kainate (\(n = 35\)), and quantitatively there was a more than 3-fold increase in the ratio of glutamate to kainate-evoked currents for GluR1-Lc channels (\(I_{\text{glutamate}}/I_{\text{kainate}} = 0.26 \pm 0.03\), wild type; \(I_{\text{glutamate}}/I_{\text{kainate}} = 0.78 \pm 0.05\) GluR1-Lc; \(n = 4\)).

**Fig. 1.** Left panel, schematic representation of a glutamate receptor ion channel. The region signified as M2 was initially thought to be a transmembrane region, but recent topology models indicate that it forms a re-entrant loop and contributes to the lining of the pore. The Lurcher mutation (\(star\)) is located near the M3 region. Right panel, NCBI BLAST alignment of the amino acid sequence around the Lurcher site in the GluR62 subunit. Only differences in amino acids are designated by letter codes.

**Fig. 2.** A, time-dependent changes of the inward currents recorded from oocytes expressing wild-type GluR1 or GluR1-Lc. Inward current was recorded upon clamping the oocytes to –60 mV. B and C, example traces showing the currents induced by kainate (100 \(\mu\)M) or glutamate (1 mM) in oocytes expressing GluR1-wt or GluR1-Lc channels. Kainate and glutamate were applied 10 min after the electrode penetration when the initial inward current reached a relatively steady level. In oocytes expressing GluR1-Lc, both agonists induced larger inward currents as well as a slow inactivation. D, E1, and E2, current-voltage (I-V) relationships of kainate-induced or agonist-independent inward currents (spontaneous) in oocytes expressing GluR1-wt (\(n = 5\)) or GluR1-Lc (\(n = 5\)), respectively. Currents were not leak-subtracted. Kainate-induced currents from GluR1-wt oocyte and the spontaneous current from GluR1-Lc receptor were eliminated following the substitution of nonpermeable ions NMDG. The amplitude of spontaneous current from GluR1-Lc receptor decreases gradually with time (E1 and E2). F, kainate (100 \(\mu\)M)-induced currents in oocytes expressing GluR1-wt and GluR1-Lc were sensitive to antagonism by GYKI 52466 (40 \(\mu\)M). Note the much shorter time scale of these recordings compared with panel C and the initial peak carried by Ca\(^{2+}\)-activated chloride currents. G, GYKI 52466 also depressed the spontaneous current in oocytes expressing GluR1-Lc. The lower sensitivity of GluR1-Lc currents to this antagonist may reflect the much higher potency of kainate for GluR1-Lc receptors.
The spontaneous current present in oocytes expressing GluR82-Lc can be suppressed by substituting Na⁺ and K⁺ with the impermeant cation NMDG. The initial inward current recorded from oocytes expressing GluR1-Lc channels in the absence of applied agonist was also suppressed (Fig. 2E1) by this substitution (Fig. 2, E1 and E2). We also tested the noncompetitive AMPA channel blockers GYKI 52466 (n = 6) and N-(4-hydroxyphenylpropanol)spermine (HPP-SB; not shown) (11) against this inward current. Applications of GYKI 52466 depressed this inward current (Fig. 2G), confirming that GluR1-Lc channels mediated the initial inward current observed in the absence of applied agonist (n = 45). Applications of these blockers also reversibly depressed kainate-evoked responses (Fig. 2F). The potency of GYKI 52466 appeared to be less for GluR1-Lc than for responses of GluR1-wt channels; however, this observation must take into account the substantial increase in the potency of kainate observed for GluR1-Lc responses (see below).

We next examined the possible mechanism of the slow inactivation of currents in oocytes expressing GluR1-Lc channels. Agonist-induced inactivation is shown quantitatively in Fig. 3, A and B; and we initially considered that it might depend upon extracellular Ca²⁺. Extracellular Ca²⁺ was therefore replaced with Ba²⁺, a cation that usually does not substitute for Ca²⁺ in inactivation of channels. This substitution reversibly blocked the slow inactivation observed in oocytes expressing GluR1-Lc channels (Fig. 3, C and D) (n = 20). Ba²⁺ substitution also prevented the slow inactivation of the initial inward current. Oocytes were then injected with EGTA for the purpose of chelating Ca²⁺ entering through the GluR1-wt and GluR1-Lc channels. Injections of EGTA did not obviously alter kainate responses in oocytes expressing GluR1-wt but blocked the slow inactivation of kainate-evoked currents observed for GluR1-Lc channels. The influx of Ca²⁺ through GluR1-wt and GluR1-Lc channels also causes a secondary activation of Ca²⁺-dependent Cl⁻ currents (12, 13). These Cl⁻ currents are inward under our recording conditions, inactivate rapidly, and contribute to the peak seen at the beginning of agonist application (see Fig. 2F) (12, 13). Therefore, these currents are unlikely to be responsible for the observed slow inactivation of GluR1-Lc-mediated currents.

To determine whether the Lurcher mutation had altered binding and/or gating of the receptors, we constructed concentration-response relationships for kainate and glutamate. These were done in Ba²⁺-substituted solution to prevent slow inactivation (Fig. 4, A and B). In comparison with oocytes expressing GluR1-wt channels, those expressing GluR1-Lc demonstrated responses that were more than 80 times more sensitive to kainate (GluR1-wt; EC₅₀ = 71 μM; GluR1-Lc, EC₅₀ = 0.8 μM) and almost 2000-fold more sensitive to glutamate (GluR1-wt; EC₅₀ = 331 μM; GluR1-Lc, EC₅₀ = 0.2 μM). It should be noted that our EC₅₀ value for glutamate-evoked currents from GluR1-wt channels cannot simply be equated to the affinity of the desensitized state of the receptor, as it also reflects the kinetics of recovery and re-entry into desensitized states.

The dramatic increase in the potency for glutamate of GluR1-Lc suggests that contaminating glutamate in the extracellular solution might have been sufficient to activate these channels and generate inward currents. For this reason, free concentrations of glutamate in our bathing solutions before and after electrode penetration of oocytes were measured in a static bath using high pressure liquid chromatography. Final bath concentrations of glutamate were below detectable values in our extracellular solutions but increased to measurable values following electrode insertion (35–150 nm). Therefore, glutamate is released upon electrode insertion, and concentrations would be sufficient to activate GluR1-Lc channels.

During experiments designed to determine the potency of the competitive AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), we were surprised to discover that applications of the antagonist itself activated GluR1-Lc channels (Fig. 4). As anticipated, this compound antagonized responses of GluR1-wt to kainate (Fig. 4C) and glutamate without activating currents on its own. In contrast, CNQX-evoked currents for GluR1-Lc channels demonstrated similar properties of rectification, as did responses to glutamate and kainate (Fig. 4D). Responses to CNQX were also blocked by GYKI 52466 (not shown), and CNQX appeared more potent (EC₅₀ = 53 nm) as an agonist of GluR1-Lc receptors than kainate itself. To determine whether the properties of GluR1-Lc channels were dependent upon the expression system (i.e., oocytes), we also examined responses to kainate and CNQX in HEK293 cells transfected with GluR1-wt or GluR1-Lc cDNAs. Kainate-evoked currents in cells expressing GluR1-wt were blocked by applications of CNQX, and CNQX never evoked any inward current on its own (n = 10). However, as we had observed with oocytes, CNQX or kainate applications both

FIG. 3. Ca²⁺ dependence of the slow inactivation of kainate-evoked currents in oocytes expressing GluR1-Lc. A, representative traces showing glutamate-evoked and kainate-induced currents from oocytes expressing GluR1-wt or GluR1-Lc. Inward current from GluR1-wt remains relatively stable, whereas the current from GluR1-Lc demonstrates a characteristic time-dependent inactivation. B, summary data showing the time-dependent change of kainate or glutamate-induced currents from oocytes expressing either GluR1-wt or GluR1-Lc (n = 4–7 oocytes or each treatment). C, example traces showing the absence of slow inactivation of kainate-induced current when extracellular Ca²⁺ was replaced by Ba²⁺. The inactivation was restored upon switching back to Ca²⁺-containing solution. D, summary data showing the lack of slow inactivation of kainate-induced currents in Ba²⁺-containing solution. E, pre-injection of oocytes with EGTA (50 nl of 100 mM, final concentration estimated to be ~5 mM) also abolished the slow inactivation of kainate-induced currents from oocytes expressing GluR1-Lc in the presence of extracellular Ca²⁺.

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evoked inward currents in cells expressing GluR1-Lc (Fig. 4F) (n = 8). In contrast to recordings in oocytes, we failed to record any spontaneous inward currents from HEK293 cells expressing GluR1-Lc channels.

In summary, GluR1-Lc-mediated currents demonstrated a slow inactivation in response to prolonged applications of agonist that was not seen with GluR1-wt channels. GluR1-Lc channels also mediated the initial inward current, and this current slowly inactivated in the absence of applied agonist. Applications of agonist also accelerated the slow inactivation of the initial inward current, and both forms of inactivation were dependent upon an influx of Ca\(^{2+}\) through GluR1-Lc channels, as the removal of extracellular Ca\(^{2+}\) and enhanced chelation of intracellular Ca\(^{2+}\) reduced both.

Even more striking were the dramatic increases in the potency of both kainate and glutamate for GluR1-Lc receptors as well as the conversion of the competitive antagonist CNQX into a potent agonist. Both of these actions are strikingly similar to results reported for single-site mutations within the second transmembrane region of the α7 cholinergic receptor (14). Point mutations converted antagonists to agonists and also increased the potency of agonist. It was suggested that such mutations stabilize the desensitized forms of the receptor in an open channel conformation. Such an interpretation is consistent with our observations that GluR1-Lc channels are much more sensitive to agonist, perhaps reflecting the much higher affinity of the desensitized state. Whether these changes primarily reflect alterations in the binding of the agonist to the receptor or a change in gating remains to be determined (15).

The high potency of glutamate (EC\(_{50}\) = 170 nM) for GluR1-Lc channels together with the release of glutamate from oocytes may account for our observation of the initial inward current. This interpretation was supported by the lack of such currents in HEK293 cells expressing these channels. It seems reasonable to speculate that the GluR82 Lurcher mutation might also demonstrate similar high potency for glutamate. Ambient concentrations of glutamate (16) in vivo would then be sufficient to continuously activate mutant channels.

The Lurcher mutation occurs in a region in or adjacent to channel pore and links the pore with the extracellular loop containing the S2 segment. Activation of AMPA and kainate receptors by glutamate is thought to involve an initial binding of this amino acid to the S1 segment in the N terminus, followed by interactions with the S2 segment of the extracellular loop (17). Upon the binding of glutamate, the two lobes of the channel formed by the S1 and S2 segments may move together, perhaps accounting for agonist trapping and desensitization (18). A single-site mutation in the S1 region of GluR3 (L507Y) is sufficient to eliminate desensitization without changing most other properties of binding and channel gating (19). This finding implies that an interaction of agonist with the S1 region is sufficient for both agonist binding and channel gating. It seems reasonable to suggest that in GlurR32 and AMPA receptors, an alamane that lies at a key and homologous site near the second transmembrane region is critical for maintaining the physical relationships between agonist binding and channel gating.

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