Dominant negative mutant of ionotropic glutamate receptor subunit GluR3: implications for the role of a cysteine residue for its channel activity and pharmacological properties

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We reported that a 33-amino-acid deletion (from tyrosine-715 to glycine-747) in a putative extracellular loop of GluR3 produced a mutant that exhibited dominant negative effects upon the functional expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [Sekiguchi et al. (1994) J. Biol. Chem. **269**, 14559–14565]. In this study, we searched for a key residue in the dominant negative effects to explore the mechanism and examined the role of the residue in the function of the AMPA receptor. We prepared 20 GluR3 mutants with amino acid substitutions within the 33-amino-acid-region, and dominant negative effects were tested electrophysiologically in *Xenopus* oocytes co-expressing the mutant and normal subunits. Among the mutants, only a GluR3 mutant in which an original cysteine (Cys)-722 was replaced by alanine exhibited a dominant negative effect comparable with that of the original mutant in which the entire 33-amino-acid segment is deleted. The co-expression of the Cys-722 mutant did not inhibit the translation of normal subunits in oocytes. The Cys-722 mutant formed a functional homomeric receptor with significantly higher affinity for glutamate or kainate than a homomeric GluR3 receptor. The Cys-722 mutation greatly enhanced the sensitivity of GluR3 for aniracetam, which alters kinetic properties of AMPA receptors. The kainate-induced currents in oocytes expressing the Cys-722 mutant alone showed strong inward rectification. These results suggest that the Cys-722 in GluR3 is important for dominant negative effects and plays a crucial role in the determination of pharmacological properties in AMPA receptor function.

INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system and its fast actions are mediated through ionotropic glutamate receptors. These are divided into three subtypes, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors [1–3]. The AMPA receptor is thought to consist of four subunits, named GluR-1, -2, -3 and -4 [4]. Each subunit is expressed in two or more variants generated by alternative splicing and RNA editing [5,6]. Native AMPA receptors are believed to function as oligomeric complexes of these subunits, most likely as pentamers [7–9].

Structure–function relationships of recombinant AMPA receptors have been intensively investigated. With regard to transmembrane topology, a three-transmembrane-domain (TMD) topology model for AMPA receptors, which differs from the previous four-TMD model, was proposed as a result of analyses using *N*-glycosylation site tagging and insertional mutation of antigenic epitopes [10,11]. This model gives one putative extracellular loop domain between TMDs-B and -C, which consists of about 173 amino acids for GluR3. This domain has been of particular interest because it contains sites that can affect the behaviour of the AMPA receptor. Namely, the domain contains the alternative splicing segment for flip and flop variants [5], one of the structural determinants for allosteric modulation by cyclothiazide [12], a phosphorylated site [13] and a site that undergoes an RNA editing (for GluR-2, -3, and -4) [6]. Furthermore, it was suggested that this domain is involved in agonist binding together with the N-terminal domain [14,15]. In the NMDA receptor, two cysteine residues in the corresponding region are required for redox modulation of the receptor [16].

We reported that a GluR3 mutant (sGluR3) carrying a 33-amino-acid deletion (from Tyr-715 to Gly-747, see Figure 1) in the putative extracellular loop of GluR3 no longer functions as an active receptor, and that sGluR3 acts as a dominant negative mutant for the functional expression of AMPA receptors by forming non-functional complexes with normal AMPA receptor subunits [17]. In that study, however, we could not analyse in detail how the mutation might contribute to the mechanism of the dominant-negative phenotype, since sGluR3 itself was non-functional. To explore this question, we decided to identify residues that are important for the dominant negative effects, and to examine their role in the function of AMPA receptors. In this study, we prepared GluR3 mutants having amino acid substitutions within the 33-amino-acid-region, and the dominant negative effects were tested electrophysiologically in *Xenopus* oocytes co-injected with the mutant and normal subunit mRNAs. We found that one mutant in which cysteine (Cys)-722 was replaced by alanine in GluR3 had a dominant negative effect upon the functional expression of normal AMPA receptors. Although the activity was weaker than that of GluR3, the Cys-722 mutant formed a functional homomeric receptor. The Cys-722 mutation increased the apparent affinity of GluR3 for glutamate or kainate and enhanced the sensitivity of GluR3 for aniracetam, which changes the kinetic properties of AMPA receptors. These results suggested the importance of the original cysteine residue in defining the fundamental pharmacological

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; TMD, transmembrane domain.

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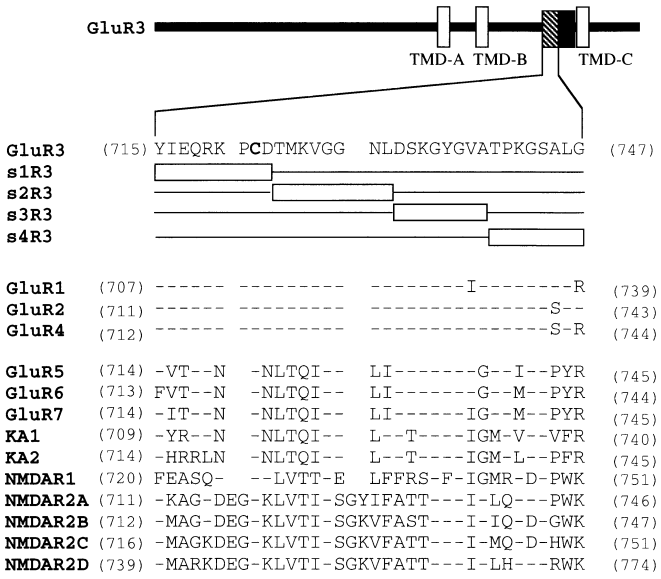

Figure 1 A schematic drawing of the protein structure of GluR3

The shaded and solid portions show the positions of the region in which a deletion impaired the receptor function and the position of the flip-flop segment, respectively. Open boxes at the top of the Figure represent putative transmembrane domains (TMD) in the three topology model [10]. The amino acid sequence in the region is denoted by an amino acid single-letter code for all GluRs. The residue that is common to GluR3 is indicated by a dash and a gap in the sequence is indicated by a blank. In s1R3, s2R3, s3R3 and s4R3, deleted segments are shown below with the deleted area as a white box.

properties of AMPA receptors and provided insights into structure–function relationships of AMPA receptors. The mutated GluR3 are sure to serve as molecular tools for revealing the physiological roles of AMPA receptors *in vivo*.

MATERIALS AND METHODS

Construction of mutants

The cDNAs encoding GluR1-flop and GluR3-flop inserted into the plasmid Bluescript SK^- and those encoding GluR6, KA2, NMDAR1 (NR1) and NMDAR2B (NR2B) inserted into the plasmid Bluescript KS^+ were provided by Dr. S. Heinemann, The Salk Institute, San Diego, CA, U.S.A. The numbering system counts from the N-terminus of the putative mature proteins (excluding a putative signal sequence in the N-terminus). Site-directed mutagenesis proceeded as described [17] using a Muta-Gene Phagemid kit (Bio-Rad), and the incorporation of desired mutations was confirmed by analysis with an A.L.F. DNA sequencer (Pharmacia). Point mutations are referred to using conventional single-letter nomenclature, for example, R3C722A, where C is the original cysteine residue at position 722, A is a mutated alanine residue, and R3 means GluR3.

Injection and electrophysiology in *Xenopus* oocytes

Synthesis and injection of cRNA into oocytes and electrophysiological recordings from the oocytes were performed as previously described [17]. Briefly, the cRNA to be injected into oocytes was prepared from linearized cDNA by transcription *in vitro* using the mMessage-mMachine kit (Ambion), and the concentration of the cRNA samples was adjusted to 1.0 μg/μl by measuring the absorbance at 260 nm. Solutions containing cRNAs for normal GluR and variants were prepared by mixing the cRNAs prior to injection (the ratio = 1:4 unless otherwise specified). Solutions containing cRNAs for normal GluRs only, which were injected in parallel as controls, were mixed with distilled water before injection. The average volume injected was $\sim$ 50 nl. After a 3–5 day incubation, electrophysiological responses were measured from oocytes superfused with frog Ringer's solution (115 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes, pH 7.2) using a conventional two-electrode voltage-clamp method. All reagents were dissolved in the frog Ringer's solution and applied to oocytes with superfusion. To record the responses of kainate receptors to kainate, oocytes were incubated with 0.3 mg/ml concanavalin A for $>$ 3 min prior to recordings. The responses in oocytes expressing NMDA receptors were recorded in frog Ringer's solution in which $CaCl_2$ was totally replaced by 1.8 mM $BaCl_2$ [18]. Kainate and glutamate were purchased from Wako Pure Chemicals. Aniracetam was obtained from Sigma. In seven or more oocytes expressing wild-type subunits alone or those co-expressing mutant subunits with them, the amplitude of the response to 100 μM kainate in the control and the test was measured at -70 mV, and the values were averaged in each group. The mean value obtained from mutant-co-expressing oocytes was normalized by that obtained from wild-type-expressing oocytes. The normalized value is represented as the 'relative amplitude'. These experiments were repeated in oocytes prepared from other frogs (at least three times), and the values of the 'relative amplitude' were averaged.

Immunoprecipitation

Labelling of GluR1 by [^{35}S]methionine, immunoprecipitation with anti-peptide antibodies specific for GluR1, and analyses on SDS/PAGE proceeded as described [17]. Briefly, three oocytes injected with cRNA were incubated in the modified Barth's medium containing [^{35}S]methionine (1 mCi/ml) and homogenized after 72 h. The homogenate was precipitated with immobilized antibodies specific for GluR1 [8], then resolved by SDS/PAGE. The gels were dried and proteins revealed by autoradiography.

Assay for cell surface localization

Cell surface localization of wild-type and mutant proteins was investigated by using cell-surface biotinylation as described by Stern-Bach et al. [14]. At 5 days after injection of cRNAs ($\sim$ 25 ng each per oocyte), six to ten oocytes were incubated with or without 1 mg/ml NHS-SS-Biotin (Pierce) for 4 h at 4 °C. After extensive washing with an excess of cold buffer, four to seven intact oocytes were homogenized in solubilization buffer [20 mM Tris/HCl (pH 7.4)/200 mM NaCl] and solubilized by adding Triton X-100 to a final concentration of 1.5 %. After 1 h of incubation on ice, homogenates were centrifuged and the supernatants were collected. A portion was saved as the Total fraction (T), and the rest was precipitated with streptavidin beads (Pierce) at 4 °C for 2 h. After extensive washing, the beads were incubated for 15 min with loading buffer [0.13 M Tris/HCl (pH 6.8)/10 % glycerol/2 % SDS/150 mM dithiothreitol], and then removed by centrifugation. The supernatant was collected as the Pellet fraction (P). Each fraction was analysed by SDS/8 %-(w/v)-PAGE and immunoblotted with antibodies specific for GluR2 and GluR3 [8]. The percentages of the proteins detected in the membrane were estimated by measuring the density of each band [14]. The density of the band obtained in the P fraction in the presence of biotin was divided by that in the corresponding T fraction while taking into account the dilution

factor for each fraction. Then, from the value obtained, that obtained from the bands in the absence of biotin (= non-specific binding fraction) was subtracted.

Pharmacological analysis

The data of the concentration response curves were fitted to the logistical function $I = I_{max}/[I+(ED_{50}/A)^h]$, where I is the current response, I_{max} is the maximum response, ED_{50} is the concentration causing a 50% maximal response, A is the concentration of agonist, and h is the Hill coefficient. Data obtained from several oocytes were averaged and expressed as means ± S.E.M.

Statistics

Differences between two experimental groups were statistically analysed by Student's t test. Since the values of 'relative amplitude' in oocytes expressing mutants and normal subunits alone (= 100%) could not be statistically compared, we compared the raw values (nA) obtained in each injection group using Student's t test.

RESULTS

To search initially for a residue that is important in conferring dominant negative effects, we examined the 33 amino acid segment in detail. The segment was divided into four, and cDNAs encoding four mutants in which each was deleted in GluR3 were prepared (s1R3, s2R3, s3R3 and s4R3; Figure 1). The s1R3 mutant lacks Tyr-715 to Asp-723; the s2R3 mutant lacks Thr-724 to Leu-731; the s3R3 mutant lacks Asp-732 to Ala-739; and the s4R3 mutant lacks Thr-740 to Gly-747. The cRNAs transcribed from the mutated cDNAs were injected into oocytes together with the cRNA for normal GluR3. After a 3–5-day incubation, the amplitude of kainate-induced currents was compared between oocytes expressing GluR3 alone and GluR3 plus one of the deletion mutants. Under these condition, oocytes injected with the mutated cRNAs alone did not respond to 100 μM kainate. As Figures 2(A) and 2(B) show, the kainate responses in oocytes expressing GluR3 + s1R3 or s4R3 (see Figure 1) were much smaller than those in oocytes expressing GluR3 alone. The amplitude of the kainate responses in oocytes expressing GluR3 + s2R3 or s3R3 (Figure 1) and those in oocytes expressing GluR3 alone did not differ. These results suggested that the regions deleted in s1R3 and s4R3 are especially important for dominant negative activity, whereas those deleted in s2R3 and s3R3 are not essential.

To survey amino acid residues which are important for dominant negative effects, we replaced each residue in regions lacking in s1R3 and s4R3 with Ala (a residue which was originally Ala was replaced by Arg; see Figure 3B) and the mutants were expressed with or without wild-type GluR3. When these mutants were expressed alone, we found that ten of the 17 mutations (Y715A, E717A, P721A, C722A, D723A, T740A, P741A, K742A, L746A and G747A) outstandingly impaired ion-channel activity of GluR3. On the other hand, in several mutants, such as R3K720A, the size of the response to 100 μM kainate was not significantly changed. When co-expressed with GluR3, we found that R3C722A and R3L746A significantly lowered the amplitude of the response of oocytes to kainate. As shown in Figure 3(A), the response of oocytes to kainate is greatly suppressed by co-expression of R3C722A. The mean amplitude of the kainate-evoked current fell to 21% of that from oocytes expressing GluR3 alone [Figure 3B(a)]. In R3L746A, the relative amplitude was slightly decreased to 67% [Figure 3B(b)]. Other mutants did not show significant negative changes in the amplitude of the response to kainate. These results suggest that replacing Cys-722 and Leu-746 with Ala produced mutants which suppress the functional expression of GluR3, and that mutation of Cys-722 gives much more potent suppressing activity than mutation of Leu-746.

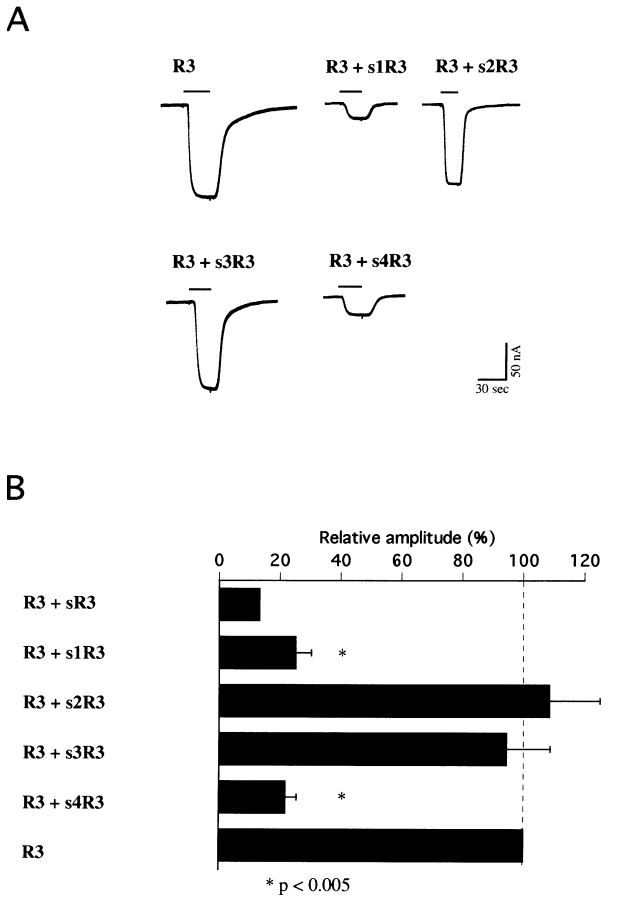

Figure 2 s1R3 and s4R3 inhibit functional expression of GluR3

(**A**) Sample records obtained from oocytes injected with the cRNA for GluR3 (10 ng), GluR3 (10 ng) + s1R3 (40 ng), and GluR3 (10 ng) + s4R3 (40 ng). All records were obtained at a holding potential of −70 mV, and a kainate (K) concentration of 100 μM. In this and subsequent Figures, the downward deflection reveals inward current, and all the reagents were applied by superfusion in frog Ringer's solution for the period indicated by horizontal bars. (**B**) Summary of results from oocytes obtained from at least four frogs per tested combination; GluR3 (10 ng) + s1R3 (40 ng), GluR3 (10 ng) + s2R3 (40 ng), GluR3 (10 ng) + s3R3 (40 ng), GluR3 (10 ng) + s4R3 (40 ng) and GluR3 (10 ng). The mean amplitude of each combination was normalized to the mean response in the oocytes expressing GluR3 alone for each individual injection, and the values obtained from four to six injections were averaged. As a positive control, the mean response obtained from a single experiment using several oocytes injected with cRNAs for GluR3 (10 ng) + sGluR3 (sR3) (40 ng) is indicated. Error bars show mean ± S.E.M. Differences of the raw data (nA) obtained from at least four experiments were statistically analysed by Student's t test.

To investigate whether replacement of Cys-722 and Leu-746 with an amino acid other than Ala also produces mutants suppressing functional expression of GluR3, we prepared two mutants in which arginine was introduced to these residues. R3C722R exhibited significant suppressive action upon functional expression of GluR3 [Figure 3B(a)], although the extent of the suppression was weaker than that of R3C722A. R3L746R did not show significant inhibitory action when co-expressed with GluR3. These results suggest that the replacement of Cys-

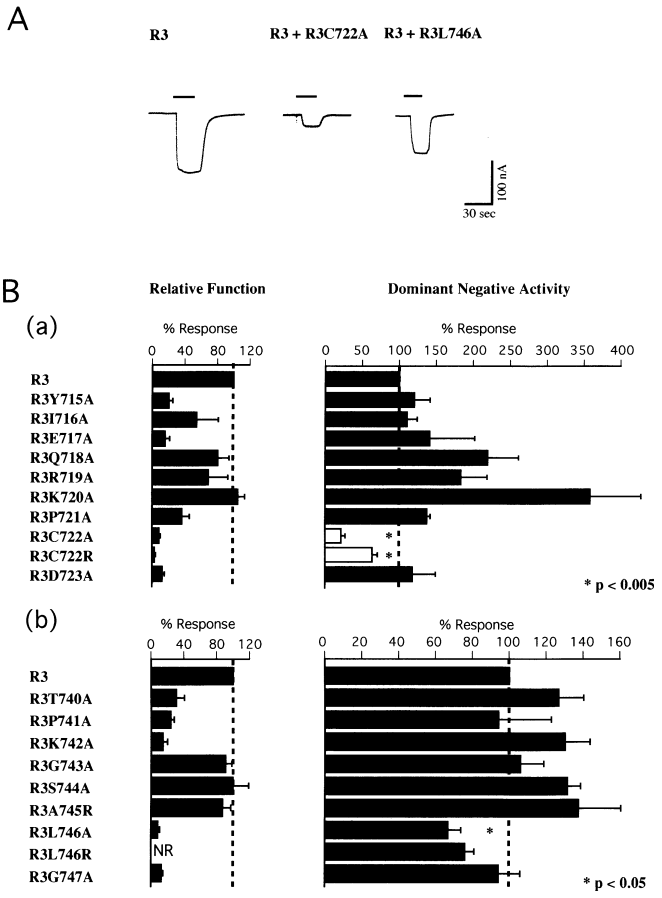

Figure 3 A single amino acid substitution in GluR3 also causes the dominant negative phenotype

(**A**) Sample records from oocytes injected with cRNA for GluR3 (10 ng), GluR3 (10 ng) + R3C722A (40 ng) and GluR3 (10 ng) + R3L746A (40 ng). (**B**) Left column: the effect of expression of each substituted mutant in residues 715–723 (**a**) and that in 740–747 (**b**), respectively, on the size of the kainate response. The mean amplitude of the kainate responses (100 μM, at -70 mV) obtained from seven to ten oocytes injected with cRNAs for denoted mutant (10 ng) is expressed as a percentage of the mean amplitude obtained from oocytes injected, in parallel, with the cRNA for GluR3 alone (10 ng). The values are averages of three to ten experiments and the means $\pm$ S.E.M. NR (not responsive) indicates that kainate application (100 μM) did not elicit any significant response in at least ten oocytes. Right column: the effect of co-expression of each substituted mutant on the size of the kainate response. Current recordings obtained from oocytes with cRNAs for GluR3 (10 ng) and denoted mutant (40 ng) are normalized to the mean response in the oocytes injected with cRNAs for GluR3 alone (10 ng) for each individual injection, and the values are the average of four to ten injections. Error bars show S.E.M.

722 is more directly involved in dominant negative effects than that of Leu-746. The fact that the dominant negative action of R3C722A was similar to the effects of the original dominant negative mutant (sGluR3) [17] also seems to support this notion. Therefore, we further analysed R3C722A.

The properties of the R3C722A suppressive actions were compared with those of sGluR3. As reported, sGluR3 exhibited dominant negative effects upon the functional expression of GluR3 and other subunits of the AMPA receptors [17]. As Figure 3(A) and Figure 4(A) show, R3C722A suppressed functional expression of GluR3, GluR1 and hetero-complexes formed from GluR1, GluR2 and GluR3. We also showed that the deletion of a 33-amino-acid segment corresponding to the segment deleted in sGluR3 made a dominant negative GluR1 mutant [17]. Figure 4(A) shows that a GluR1 mutant in which Cys-714, a residue corresponding to Cys-722 of GluR3, was replaced by Ala, suppresses the functional expression of GluR1.

Since co-expression of sGluR3 did not inhibit the translation of normal subunits [17], we examined whether the co-expression of R3C722A inhibits the translation of normal subunits. Oocytes co-injected with cRNAs for GluR1, GluR1 + GluR3, and GluR1 + R3C722A were incubated with [^{35}S]methionine and homogenized. The homogenate was incubated with immobilized antibodies specific for GluR1 [8] and the materials specifically bound to the antibodies were analysed on SDS/PAGE. As Figure 4(B) shows, one intense radioactive band was seen in all the samples from oocytes co-expressing GluR1 (lane 2, GluR1; lane 3, GluR1 + GluR3; lane 4, GluR1 + R3C722A; lane 1 is a water-injected control), and there was no outstanding difference in the abundance of the labelling between the three conditions in which GluR1 cRNA was injected. These results indicated that the properties of the R3C722A dominant negative effects resembled those of sGluR3.

One plausible mechanism for dominant negative actions of R3C722A and sGluR3 is that these mutants act by assembling with normal AMPA receptor subunits and thereby reducing the trafficking of the channel to the cell surface. To address this possibility, cell-surface expression of mutants and wild-type proteins was analysed using cell surface biotinylation. As shown in Figure 4(C), the percentage of the subunit proteins which reach the cell surface was similar for wild-type R3, sGluR3 and R3C722A ($\sim 10\%$). These results suggested that R3C722A and sGluR3 were properly transported to the cell surface, and co-expression of these mutants with wild-type subunits would not affect the trafficking of the channel to the cell surface. As shown in Figure 3(B), oocytes injected with cRNAs of R3D723A alone responded to kainate as weakly as those injected with cRNAs of R3C722A alone, but R3D723A showed no inhibitory phenotype. The mutants like R3D723A may have greatly reduced ability of oligomerization and, as a result, they would not generate the dominant negative phenotype. To address this possibility, we also examined the cell surface expression of R3D723A proteins, because unassembled proteins are often retained in the endoplasmic reticulum [19]. As shown in Figure 4(C), R3D723A also showed apparently normal surface expression. Because sGluR3, which lacks Tyr-715 to Gly-747, showed apparently normal cell surface localization, it is likely that point mutations in this region do not affect intracellular targeting of GluR3.

Cysteine is conserved at the position corresponding to Cys-722 of GluR3 in all the clones encoding rat ionotropic glutamate receptors (Figure 1). Given that the cysteine is functionally similar among iGluRs, it may be possible to transfer the dominant-negative phenotype to kainate and NMDA receptors. To address this notion, we prepared mutants of GluR6 and NMDAR1 where the conserved cysteine was replaced by alanine, and tested these mutants in oocytes. However, these mutants did not exhibit dominant negative effects on functional expression of normal GluR6 + KA2 and normal NMDAR1 + NMDAR2B, respectively (Figure 5B).

To examine the role of the Cys-722 in the function of AMPA receptors, we pharmacologically analysed oocytes injected with the cRNA alone for R3C722A. In contrast with sGluR3, R3C722A formed a functional homomeric receptor. Kainate and glutamate induced inward currents at -70 mV in oocytes expressing R3C722A alone. The amplitude of the kainate-induced currents was always smaller in oocytes expressing the mutant than in those expressing GluR3 alone. The 'relative amplitude' of kainate-induced currents calculated as described in the Materials and methods section was $8 \pm 1\%$ (Table 1). These

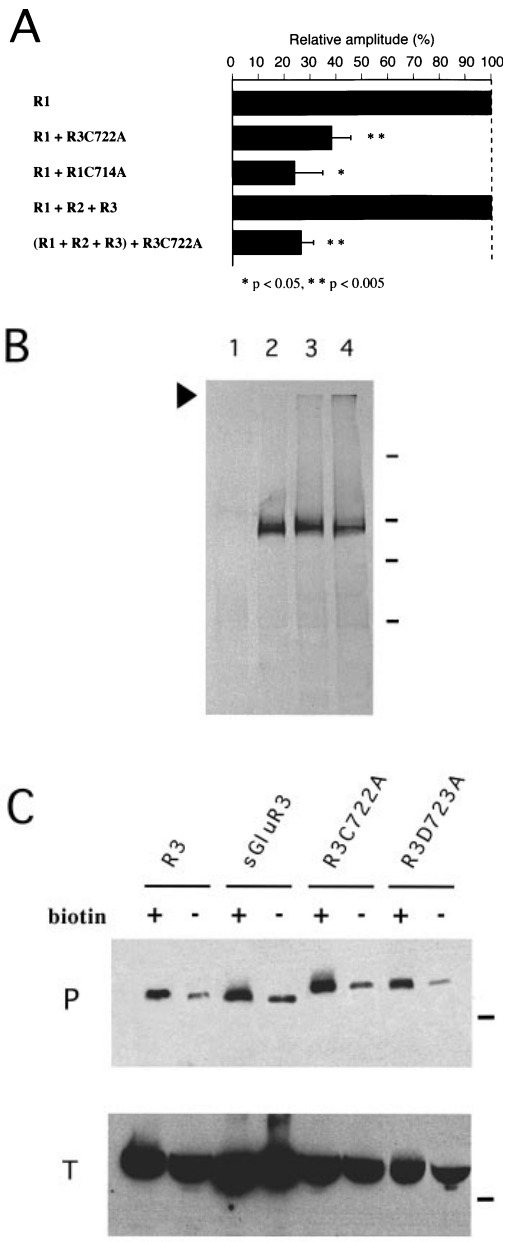

Figure 4 The properties of two AMPA receptor mutants with a cysteine-to-alanine conversion in a putative extracellular loop domain

(**A**) The effect of R3C722A or R1C714A co-expression on the functional expression of wild-type subunits of AMPA receptors. Experimental procedures were as described in the legend to Figure 2. Error bars show S.E.M. (**B**) SDS/PAGE showing immunoprecipitation of GluR1 with anti-peptide antibodies specific for GluR1. Oocytes were incubated with [^{35}S]methionine as described in the Materials and methods section. The detergent-solubilized extract was immunoprecipitated with anti-GluR1-specific antibodies and resolved by SDS/PAGE. No radioactive labelling was seen after an injection of water (lane 1), whereas an injection of GluR1 cRNA (lane 2), GluR1 plus GluR3 cRNAs (lane 3), and GluR1 plus R3C722A cRNAs all showed intense radioactive labelling corresponding to GluR1. Bars show the positions of the standards myosin (199 kDa), β-galactosidase (120 kDa), BSA (87 kDa) and ovalbumin (48 kDa). (**C**) Determination of cell-surface localization of wild-type GluR3 and mutant subunit proteins. Five days after injection of cRNAs (25 ng per oocyte), oocytes were assayed for cell surface localization as described in the Materials and methods section. Briefly, biotinylated (+) and non-biotinylated (−) oocytes were solubilized with Triton X-100, and the total solubilized proteins (T) were precipitated with streptavidin beads to separate the biotinylated proteins (P). Both fractions (20% of T and 40% of P) were separated on SDS/8% PAGE and analysed on immunoblots with antibodies specific for GluR2 and GluR3 [8]. The percentage of the proteins detected in the membrane was estimated by measuring the density of each band [14]. For wild-type GluR3 and mutants, ~ 10% of the total protein is incorporated into the membrane. Bars show the positions of the standard BSA (87 kDa).

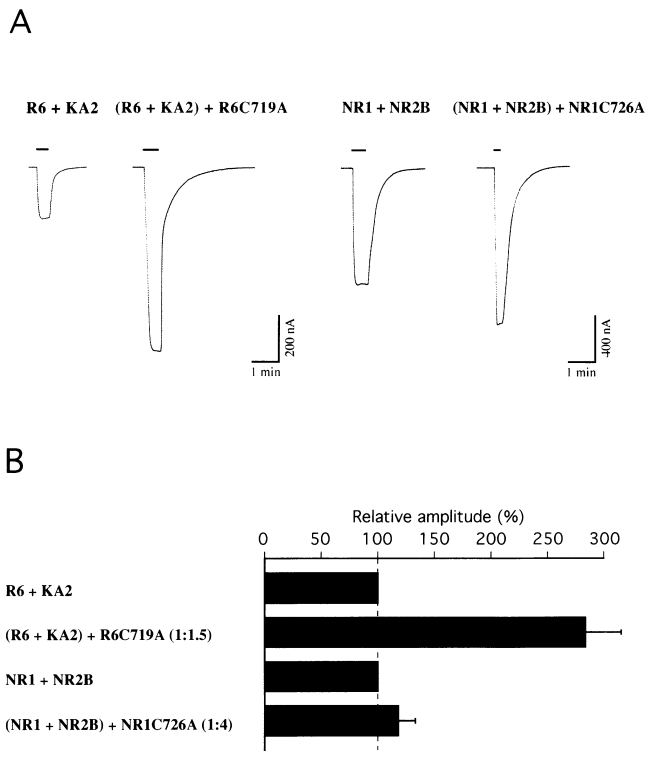

Figure 5 Neither R6C719A nor NR1C726A shows the dominant negative phenotype

(**A**) Sample records from oocytes injected with cRNAs for GluR6 (10 ng) + KA2 (10 ng), GluR6 (10 ng) + KA2 (10 ng) + R6C719A (30 ng), NR1 (5 ng) + NR2B (5 ng) and NR1 (5 ng) + NR2B (5 ng) + NR1C726A (40 ng). All records were obtained at a holding potential of −70 mV. For the NMDA receptors, the glutamate and glycine concentrations were both 10 μM. (**B**) Summary of the effects of the co-expressed homologous cysteine mutation on the size of agonist-induced responses. A similar experiment to that described in the legend to Figure 2 was performed in oocytes injected with the cRNAs indicated. Error bars show S.E.M.

Table 1 Comparison of pharmacological properties between R3 and R3C722A

Oocytes were injected with the cRNA denoted. After 3 to 6 days, the current response of oocytes to kainate (Kai) or glutamate (Glu) was recorded at −100 mV. The ED_{50} (in μM) and Hill coefficient values were calculated as described in the Materials and methods section. The efficacy of aniracetam (Ani) was calculated as follows: the amplitude of the response to glutamate (100 μM) was measured with or without aniracetam (2 mM). Each oocyte served as its own pretreatment control. The aniracetam potentiation for each oocyte was then pooled and expressed as the mean ± S.E.M. *$P <$ 0.005 when compared with the value in the wild-type.

Agonist/modulator		R3	R3C722A
Kai	ED_{50}	36.6 ± 3.4 (9)	13.9 ± 2.5 (6)*
	Hill coeff.	1.5 ± 0.3 (9)	1.5 ± 0.5 (6)
	Relative activity	1.0	0.1 ± 0.0 (6)
Glu	ED_{50}	38.9 ± 3.0 (10)	3.2 ± 0.8 (6)*
	Hill coeff.	1.2 ± 0.1 (10)	1.3 ± 0.2 (6)
Kai/Glu		7.3 ± 0.6 (22)	5.0 ± 0.2 (8)
Ani	Efficacy	7.3 ± 0.9 (7)	24.8 ± 5.8 (4)*

results suggested that the Cys-722 mutation lowered the ion-channel activity of GluR3. The dose–response curves for glutamate (Figure 6A, solid circles, GluR3; open circles, R3C722A) and kainate (Figure 6B, solid circles, GluR3; open circles, R3C722A) were shifted towards the left by the Cys-722 mutation,

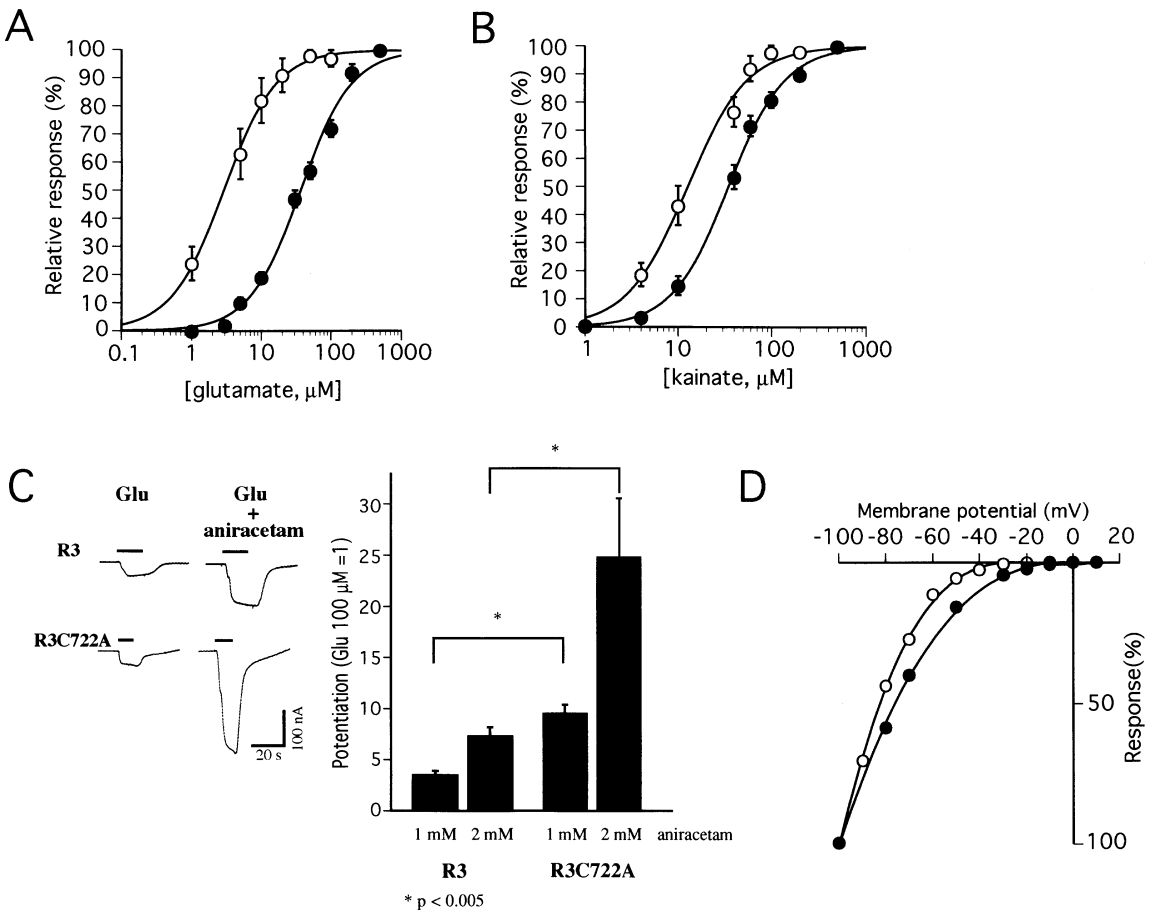

Figure 6 Pharmacological properties of R3C722A in comparison with wild-type GluR3

(**A**) and (**B**) Dose–response curves for glutamate-induced current (**A**) at −100 mV in oocytes injected with cRNAs for GluR3 (10 ng) ($n = 10$) (solid circles) and R3C722A (10 ng) ($n = 5$) (open circles), and those of kainate-induced current (**B**) at −70 mV in oocytes injected with cRNAs for GluR3 (10 ng) ($n = 7$) (solid circles) and R3C722A (10 ng) ($n = 7$) (open circles). Each value is expressed as the fraction of the mean maximal amplitude obtained by 500 μM glutamate in each injection group. (**C**) Left: Traces of glutamate (100 μM)-induced currents obtained from oocytes (−70 mV) injected with cRNA for GluR3 and R3C722A. At 5 min after glutamate application, a mixture of glutamate (100 μM) and aniracetam (1 mM) was applied to the same oocyte. Right: Potentiation by aniracetam on glutamate-induced responses in oocytes injected with the cRNA for GluR3 (50 ng) ($n = 7$) and R3C722A (50 ng) ($n = 5$). The amplitude of the response for glutamate (100 μM) plus aniracetam was normalized to that for glutamate (100 μM) alone in each oocyte. The results, expressed as potentiation on the ordinate, are the means ± S.E.M. (**D**) *I–V* curves for kainate (100 μM) response obtained in the oocytes injected with GluR3 cRNA (solid circles) or with R3C722A cRNA (open circles). The amplitude of the current at each potential is expressed as a percentage of the current at −100 mV.

suggesting that this mutation enhanced the affinity of GluR3 for glutamate and kainate. The ED_{50} values for kainate and glutamate in oocytes expressing GluR3 or R3C722A are summarized in Table 1. Furthermore, the sensitivity of R3C722A to aniracetam, which alters the kinetic properties of AMPA receptors, was compared with that of GluR3. Figure 6(C) shows that aniracetam potentiated glutamate-induced currents more in oocytes expressing R3C722A than in those expressing GluR3. These results indicate that replacing Cys-722 with Ala is not fatal, but affects the actions of glutamate, kainate and aniracetam. As seen in oocytes expressing GluR3, the current–voltage curve of kainate-induced responses showed strong inward rectification in oocytes expressing R3C722A.

DISCUSSION

This study showed that a GluR3 mutant, in which Cys-722 is substituted with Ala, exhibits dominant negative effects upon the functional expression of AMPA receptors. This Cys residue is located in the 33-amino-acid segment missing in the original dominant negative mutant, sGluR3. The levels of the dominant negative effects caused by R3C722A and sGluR3 were comparable, and the properties of the dominant negative effects of R3C722A resembled those by sGluR3. Therefore, it seems that R3C722A shares the fundamental mechanism of the dominant negative effects with sGluR3. The dominant negative phenotype caused by substituting the Cys residue was restricted to AMPA receptor subunits and the similar mutation was not effective for kainate and NMDA receptor subunits, although the Cys residue is conserved in all rat ionotropic glutamate receptors.

In the course of this work, several analyses concerning the correlation between structure and function of AMPA receptors have been reported. Studies on the transmembrane topology of AMPA receptors have concluded that the entire region between TMD-B and TMD-C is extracellular [10,11]. Stern-Bach et al. [147] analysed the chimeric receptor subunits between GluR3 and GluR6 and proposed a model for the agonist-binding of glutamate receptor subunits on the basis of the X-ray structure of bacterial periplasmic amino-acid-binding proteins[14]. According to them, the region from Tyr-715 to Gly-747 is located near the

agonist binding site, and the C-terminal half of the putative extracellular loop which contains the region is required to form a functional receptor channel complex. Furthermore, studies on the flip-flop region and its vicinity revealed that the flip and flop receptors show different desensitization [20] and that Gly-747 is involved in recovery from desensitization [6]. These recent advances are important for the basis of the following discussion about the mechanism of dominant negative phenotype and the role of the Cys residue in receptor function.

Our observations that the mutated subunits with weak or no channel activity did not always exhibit inhibitory effects provide an explanation for the molecular basis of the dominant negative effects. For example, s2R3 and s3R3 did not form a functional homomeric receptor. R3E717A, R3Y715A and R3D723A made up a homomeric receptor with weaker ion-channel activity (Figure 3B). However, none of these mutants showed inhibitory effects when co-expressed with normal GluR3-flop (Figures 2 and 3B). These observations preclude the possibility that dominant negative effects were caused by the non-specific structural alteration by the incorporation of less- or non-functional subunits into the pentameric receptor complexes, and suggest that there is a meaning in the Cys-722 mutation with respect to producing the dominant negative mutants. In other words, the Cys-722 seems to play a specific role in the receptor function.

Our pharmacological analyses assist in considering the role of Cys-722. The homomeric R3C722A exhibited higher apparent affinity for glutamate and had enhanced sensitivity for aniracetam. These results are consistent with the model by Stern-Bach et al. [14], where the C-terminal half of the region between TMD-B and TMD-C is not essentially involved in agonist binding. It is well known that aniracetam potentiates AMPA receptor currents mainly by slowing or blocking desensitization [21,22]. In outside-out patches isolated from cultured chick spinal cord neurons, AMPA receptors have an apparent affinity for glutamate 50 times higher in the desensitized state than in the closed state [23]. For all ligand-gated receptors studied to date, agonists bind to desensitized receptors with higher affinity than to resting or active receptors. These facts raise one possibility that Cys-722 is critically involved in the kinetic properties of the receptors. The kinetic model of the AMPA receptor channel complex is proposed by Patneau and Mayer [24]. In this model, there are four non-conducting states (unbound sensitized, unbound desensitized, ligand-bound sensitized, and ligand-bound desensitized states) and one conducting state (open state). Equilibrium exists between the ligand-bound sensitized and the open state receptors. Furthermore, equilibrium also exists cyclically between the four non-conducting states. The Cys-722 residue may directly or indirectly participate in one or some of the equilibria, and replacement of the Cys with Ala may shift the equilibrium toward desensitized (non-conducting) states. This would lead to the reduced amplitude of macroscopic current responses recorded in oocytes. In this concern, replacing Cys-722 with Ala alters predicted secondary structure around the Cys-722 from turn structure to α-helix (by Chou–Fasman's algorithm) and would change spatial positioning of the surrounding residues that were reported to participate in the kinetic properties of the GluR3, such as Gly-747 [6] or those in the flip-flop segment (from Asn-748 to Lys-787) [5,20]. The Cys-722 may indirectly participate in the kinetic properties by maintaining the spatial position of these residues.

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The dominant-negative GluR3 receptors will serve as powerful molecular tools to elucidate the physiological role of AMPA receptors. As shown in Figures 2 and 3, these mutants have drastic suppressive phenotype when overexpressed to wild-type AMPA receptors in oocytes. Therefore it would be possible to investigate region-specific physiological function of AMPA receptors by overexpression of R3C722A in target tissues. We generated several lines of transgenic mice which expressed a considerable amount of mRNAs of sGluR3 in brains. However, we did not detect any sGluR3 proteins by immunoblotting in them, probably due to the degradation of the proteins (results not shown). In comparison with sGluR3, which has 33 amino acids deleted, R3C722A is expected to be expressed more stably in target tissues because it has only a cysteine-to-alanine conversion. This method has a theoretical advantage over gene targeting in cases in which the genes consist of multiple members like AMPA receptors.

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