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Cloning and expression of the ε4 subunit of the NMDA receptor channel

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The primary structure of a novel subunit of the mouse NMDA (*N*-methyl-D-aspartate) receptor channel, designated ε4, has been revealed by cloning and sequencing the cDNA. The ε4 subunit shares high amino acid sequence identity with the ε1, ε2 and ε3 subunits of the mouse NMDA receptor channel, thus constituting the ε subfamily of the glutamate receptor channel. Expression from cloned cDNAs of the ε4 subunit together with the ζ1 subunit in *Xenopus* oocytes yields functional NMDA receptor channels. The ε4/ζ1 heteromeric channel exhibits high apparent affinities for agonists and low sensitivities to competitive antagonists. The ε4 subunit is thus distinct in functional properties from the ε1, ε2 and ε3 subunits, and contributes further diversity of the NMDA receptor channel.

Glutamate receptor; *N*-Methyl-D-aspartate; NMDA receptor channel; ε4 subunit; Molecular diversity; Functional expression; Pharmacological diversity

1. INTRODUCTION

The glutamate receptor (GluR) channel mediates most of the fast excitatory synaptic transmission in the central nervous system [1]. The *N*-methyl-D-aspartate (NMDA) receptor channel, one of the three major classes of the GluR channel, is highly permeable to Ca^{2+} in addition to Na^+ and K^+, and is susceptible to voltage-dependent Mg^{2+} block. These characteristics make it possible for the NMDA receptor channel to play a key role in activity-dependent synaptic plasticity in the hippocampus, thought to underlie memory and learning [2], and in experience-dependent synaptic plasticity in the developing brain [3]. In addition, abnormal activation of the NMDA receptor channel is implicated in neuronal cell death observed in various acute and chronic disorders [4,5].

Recent molecular biological studies have revealed a number of NMDA receptor channel subunits [6–10]. These subunits contain four putative transmembrane segments (M1–M4) characteristic for neurotransmitter-gated ion channels, and share amino acid sequence homology with each other and with the subunits of the AMPA- or kainate-selective GluR channel. According to the sequence homology, the NMDA receptor channel subunits are classified into the ε and ζ subfamilies of the GluR channel [7–9]. Expression of the ζ1 (NMDAR1) subunit or its smaller form (the ζ1–2 subunit) yields homomeric channels with characteristics of the NMDA receptor channel [6,7]. However, highly active NMDA receptor channels are produced only when the ζ1 or ζ1–2 subunit is expressed together with the ε1, ε2 or ε3 (NR2A, B or C) subunit [8–10]. The functional properties of the resulting NMDA receptor channels such as the apparent affinities for agonists, the sensitivities to competitive and non-competitive antagonists and regulation are critically determined by the constitute ε subunit [9]. Furthermore, each member of the ε subunit family exhibits distinct distribution in the adult brain [8–10]. Therefore, it has been concluded that the molecular diversity of the ε subunit family underlies the functional heterogeneity of the NMDA receptor channel [9]. Studies with site-directed mutagenesis have shown that the conserved asparagine in putative transmembrane segment M2 constitutes a Mg^{2+} block site of the NMDA receptor channel, and that the MK-801 site overlaps the Mg^{2+} site [11].

In the present investigation, we have revealed the presence and primary structure of a novel subunit of the mouse NMDA receptor channel. The novel subunit shares high amino acid sequence homology with the ε1, ε2 and ε3 subunits of the mouse NMDA receptor channel, and is thus designated as the ε4 subunit. The ε4 subunit, when expressed together with the ζ1 subunit, forms functional NMDA receptor channels with characteristics of high apparent affinities for agonists and low sensitivities to competitive antagonists.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APV, D-2-amino-5-phosphonovalerate; 7CK, 7-chlorokynurenate; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction.

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2. MATERIALS AND METHODS

2.1. *Cloning and sequencing of cDNAs*

Two highly conserved amino acid sequences of the mouse NMDA receptor channel subunits [7–9], WNGM(I,M)GE in the region preceding segment M1 and YTANLAA in segment M3, were chosen to synthesize the corresponding degenerate oligonucleotide primers, 5′-TGGAA(T,C)GG(A,T)AT(A,T)ATGGN(G,A)A-3′ (N = G, A, T, C) and 5′GC(G,A,T)GC(T,C)A(G,A)(G,A)TT(G,A,T)GCN(G,A)T(G,A)-TA3′. PCR was carried out for 30 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 1.5 min) after incubation at 94°C for 3 min in 50 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, ~20 ng of mouse forebrain cDNA, 2 μM each of primers, 200 μM each of four deoxyribonucleotide triphosphates and 4 U of *Taq* polymerase. The PCR products were treated with T4 DNA polymerase and inserted into the *Hin*cII site of the plasmid pBluescript II SK(+) (Stratagene). One clone carrying an ε4 subunit cDNA was identified by screening under low stringency hybridization conditions [12] and by sequencing. Screening of mouse forebrain and cerebellar cDNA libraries constructed in λgt10 under high stringency conditions with the ε4 subunit cDNA as a probe yielded several cDNA clones encoding the ε4 subunit. Additional cDNA clones were obtained by series of screenings of the same libraries with newly isolated cDNAs as probes. The cDNA inserts of these recombinant phage were subcloned into the *Eco*RI site of the plasmids pBluescript II SK(−) (Stratagene) or pBKSA [7].

The nucleotide sequences of the cDNA clones SE11 (−114 to 2,469) and SE4 (2,434 to 3,971) were determined on both strands by the dideoxy chain-termination method [13] using appropriate primers prepared with an automatic DNA synthesizer (Applied Biosystems); nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the codon specifying the amino-terminal residue of the mature ε4 subunit, and the preceding residues are indicated by negative numbers. Partial nucleotide sequences of the clones SE1 (−114 to 1,920), TSEE6 (1,635 to 2,210), and K1 (1,932 to 3,971) were in complete agreement with those of SE4 and SE11. Analysis of nucleotide and amino acid sequences was carried out using GENETYX software (SDC), and amino acid sequence identity was calculated using GeneWorks software (IntelliGenetics).

2.2. *Expression in* Xenopus *oocytes*

The entire coding sequence of ε4 subunit cDNA (residues −81 to 3,891) was inserted between the *Nco*I and *Xba*I sites of the plasmid pSP35T [14] to yield the plasmid pSPGRε4 using appropriate synthetic oligonucleotides and PCR. The ε4 subunit-specific mRNA was synthesized in vitro with SP6 RNA polymerase in the presence of 0.5 mM cap dinucleotide ^{7}mGpppG using *Eco*RI-cleaved pSPGRε4 as a template [15]. *Xenopus laevis* oocytes were injected with the ε4 subunit-specific mRNA (~16 ng/oocyte) and/or the ζ1 subunit-specific mRNA (~13 ng/oocyte) [7]. Whole-cell currents were recorded after 1–3 days incubation with a conventional two-micropipette voltage clamp [16]. Normal frog Ringer's solution contains 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES-NaOH (pH 7.2). Ba^{2+}-Ringer's solution contains 115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$ and 10 mM HEPES-NaOH (pH 7.2).

3. RESULTS AND DISCUSSION

3.1. *Cloning of the ε4 subunit*

By PCR amplification of mouse brain cDNA with degenerate oligonucleotide primers corresponding to two highly conserved sequences among the subunits of the mouse NMDA receptor channel, we found a novel member of the ε subunit family of the mouse NMDA receptor channel, designated as the ε4 subunit. Fig. 1 shows the deduced amino acid sequence of the ε4 subunit aligned with those of the ε1, ε2, ε3 and ζ1 subunits of the mouse NMDA receptor channel [7–9]. The initiating methionine is assigned to the first methionine residue found in a large open reading frame. The amino-terminal hydrophobic segment is assumed to represent a signal peptide and its cleavage site is predicted by the method of von Heijne [17,18]. The proposed mature ε4 subunit is composed of 1,296 amino acids with a calculated molecular weight of 140,656. The ε4 subunit shares 38–49% amino acid sequence identity with the members of the ε subunit family [8,9] and 13–18% identity with the subunits belonging to the other subfamily of the GluR channel subunit (Table I).

Analysis and comparison of the local hydropathicity

Table I

Percent amino acid sequence identity between rodent GluR channel subunits

Subfamily	α				β			γ		δ	ε				ζ
Subunit	α1	α2	C	D	5-1	β2	7	KA-1	γ2	δ1	ε1	ε2	ε3	ε4	ζ1
α1		66	64	66	36	37	37	34	32	25	14	13	15	15	18
α2			72	70	36	37	35	31	30	23	13	13	15	14	20
C				71	36	38	35	32	31	22	12	12	14	13	19
D					36	36	36	30	30	23	13	13	15	15	20
5-1						74	70	37	38	23	14	14	15	14	21
β2							72	39	37	21	11	12	13	13	20
7								39	39	26	13	13	15	15	21
KA-1									68	24	14	13	14	16	21
γ2										24	14	14	18	18	20
δ1											14	15	17	16	19
ε1												52	40	39	18
ε2													39	38	16
ε3														49	18
ε4															17

Sequence data are taken from [16] (mouse α1 and α2), [22] (rat C and D), [23] (rat 5-1), [24] (mouse β2), [25] (rat 7), [26] (rat KA-1), [27] (mouse γ2), [12] (mouse δ1), [8] (mouse ε1), [9] (mouse ε2 and ε3) and [7] (mouse ζ1).

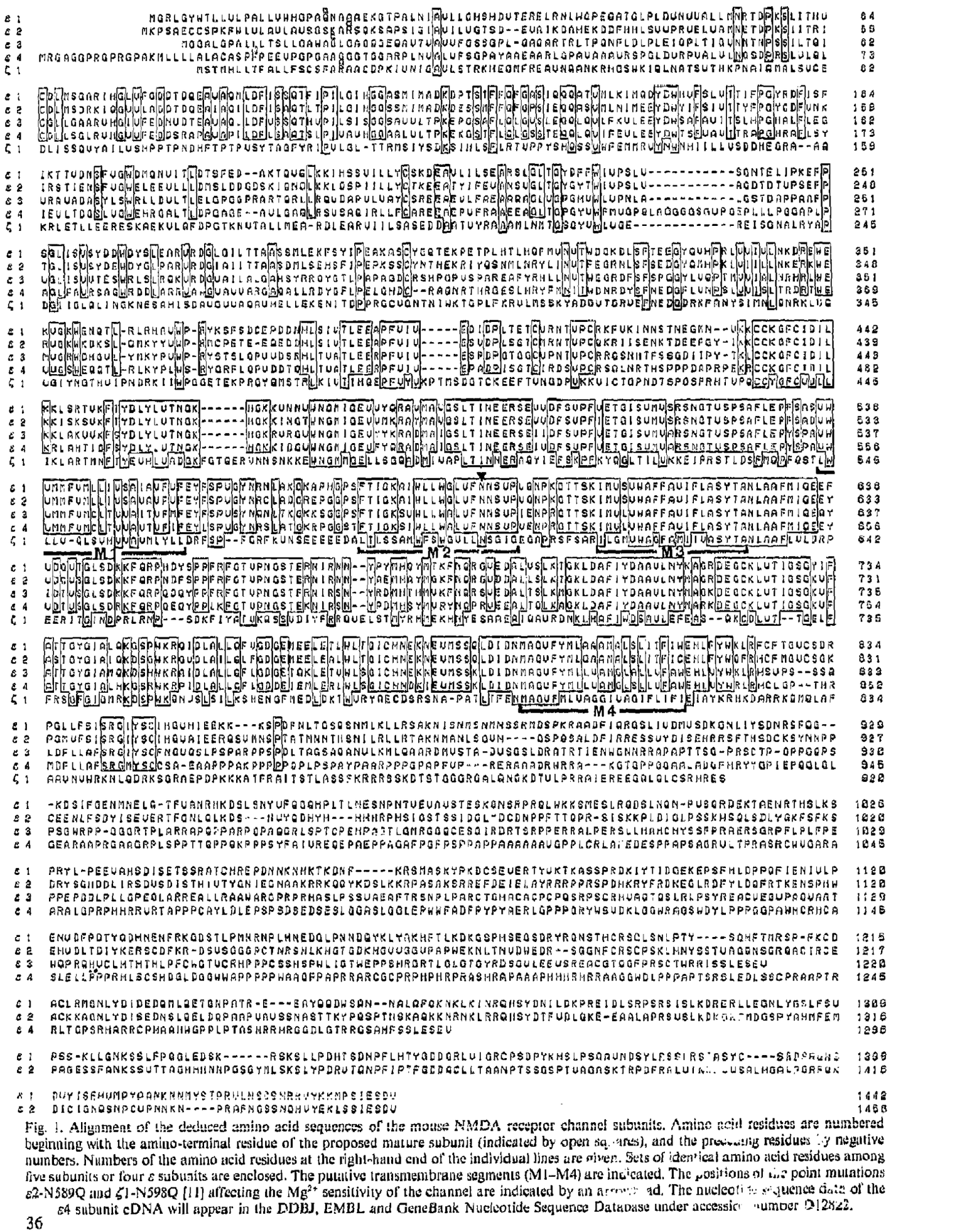

Fig. 1. Alignment of the deduced amino acid sequences of the mouse NMDA receptor channel subunits. Amino acid residues are numbered beginning with the amino-terminal residue of the proposed mature subunit (indicated by open squares), and the preceding residues by negative numbers. Numbers of the amino acid residues at the right-hand end of the individual lines are given. Sets of identical amino acid residues among five subunits or four ε subunits are enclosed. The putative transmembrane segments (M1–M4) are indicated. The positions of the point mutations ε2-N589Q and ζ1-N598Q [11] affecting the Mg^{2+} sensitivity of the channel are indicated by an arrowhead. The nucleotide sequence data of the ε4 subunit cDNA will appear in the DDBJ, EMBL and GeneBank Nucleotide Sequence Database under accession number D12822.

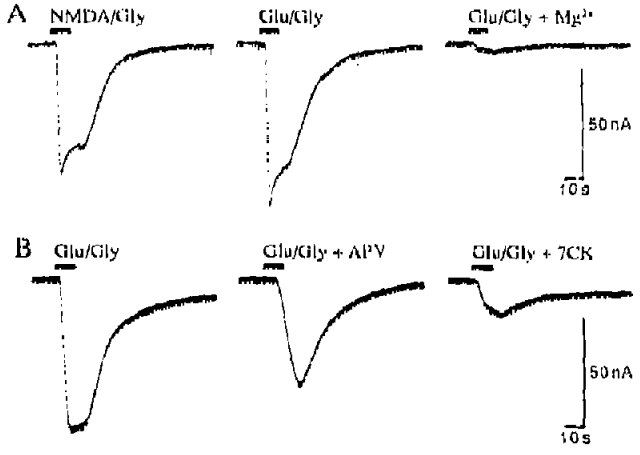

Fig. 2. Functional expression from cloned cDNAs of the $\varepsilon 4/\zeta 1$ heteromeric NMDA receptor channel in *Xenopus* oocytes. Current responses were measured at −70 mV membrane potential in normal Ringer's solution. (A) Current responses of the $\varepsilon 4/\zeta 1$ heteromeric channel to 100 μM NMDA plus 10 μM glycine (NMDA/Gly) and 10 μM L-glutamate plus 10 μM glycine (Glu/Gly), and effect of 1 mM Mg^{2+} on the response to Glu/Gly. (B) Effects of 500 μM APV and 100 μM 7CK on the response of the $\varepsilon 4/\zeta 1$ heteromeric channel to Glu/Gly.

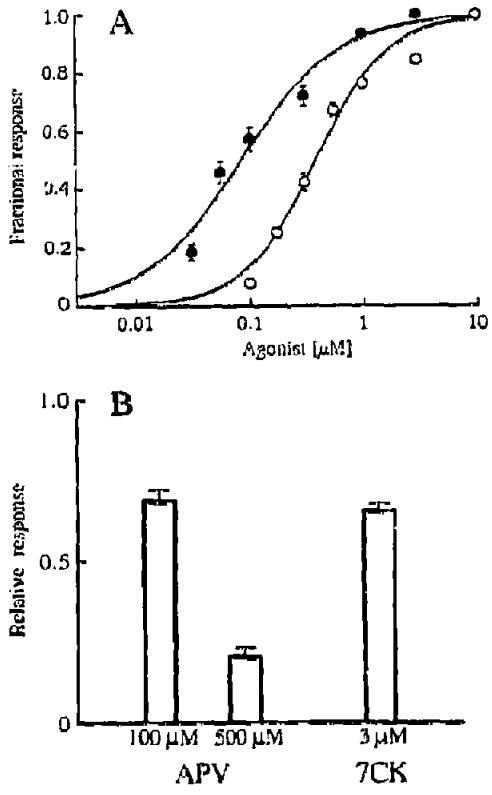

Fig. 3. Pharmacological properties of the $\varepsilon 4/\zeta 1$ heteromeric channel. Current responses were measured at −70 mV membrane potential in Ba^{2+}-Ringer's solution. (A) Dose–response relationships for L-glutamate (○) and glycine (●) in the presence of 10 μM glycine and 10 μM L-glutamate, respectively. Each point represents the mean fractional responses obtained from 4 oocytes; S.E.M. are indicated by bars when larger than the symbols. The theoretical curves have been drawn according to the equation $I = I_{max}/[1+(EC_{50}/A)^n]$, where I represents the current response, I_{max} the maximum response, A the concentration of agonist, and n the Hill coefficient. The maximum current responses were 28–43 nA (○) and 27–44 nA (●). (B) Effects of APV and 7CK on the response to 4.7 μM L-glutamate plus 0.9 μM glycine; the concentrations of agonists were ~10-fold the respective EC_{50} values. Data are presented as mean ± S.E.M. of measurements on 4–6 oocytes. Current responses in the absence of antagonists were 15–30 nA.

profile of the $\varepsilon 4$ subunit suggest the presence of four putative transmembrane segments (M1–M4) in the middle of the molecule (Fig. 1). In accordance with this is the recent finding that the asparagine residue 589 in the M2 segment of the $\varepsilon 2$ subunit, and the corresponding Asp-598 of the $\zeta 1$ subunit, constitute a Mg^{2+} block site of the NMDA receptor channel [11]. The $\varepsilon 4$ subunit contains an asparagine residue at the corresponding position in segment M2 (amino acid residue 612). In addition to the putative transmembrane regions, the region preceding segment M1 is also well conserved among GluR channel subunits, where the agonist binding site has been postulated [16,19]. Some point mutations introduced into this region of the $\alpha 1$ subunit strongly reduce the EC_{50} value for agonists of the AMPA-selective GluR channel [20,21]. Segment M4 of the $\varepsilon 4$ subunit is followed by a long carboxy-terminal sequence (461 amino acid residues), a characteristic feature common to the members of the ε subfamily [8–10]. The large carboxy-terminal region of the ε subunit, which could be either inside or outside of the membrane, may play a role in functional modulation, assembly or sorting of the NMDA receptor channel.

3.2. *Functional expression*

The $\varepsilon 4$ subunit-specific mRNA was synthesized in vitro from cloned cDNA and was injected into *Xenopus* oocytes together with the $\zeta 1$ subunit-specific mRNA. The peak inward currents obtained in normal frog Ringer's solution at −70 mV membrane potential were 70 ± 9 nA (mean ± S.E.M., n = 13) and 68 ± 13 nA (n = 8) in response to 10 μM L-glutamate plus 10 μM glycine and 100 μM NMDA plus 10 μM glycine, respectively (Fig. 2A). The current amplitudes were significantly larger than those obtained for oocytes injected with the $\zeta 1$ subunit-specific mRNA alone (17 ± 1 nA, n = 10, and 13 ± 2 nA, n = 7, respectively). The responses of the $\varepsilon 4/\zeta 1$ heteromeric channel to 10 μM L-glutamate plus 10 μM glycine were suppressed to various extents by 1 mM Mg^{2+}, 500 μM D-2-amino-5-phosphonovalerate (APV) and 100 μM 7-chlorokynurenate (7CK) (Fig. 2A,B). We thus conclude that the $\varepsilon 4$ protein is the subunit of the NMDA receptor channel.

The apparent weak suppressive effects of the competitive antagonists prompted us to examine the pharmacological properties of the $\varepsilon 4/\zeta 1$ channel quantitatively using Ba^{2+}-Ringer's solution. The EC_{50} values obtained from dose–response relationships are 0.4 μM and 0.09 μM for L-glutamate and glycine, with Hill coefficient values of 1.4 and 1.2, respectively (Fig. 3A). The apparent affinities for the agonists of the $\varepsilon 4/\zeta 1$ heteromeric

NMDA receptor channel are thus higher than the $\varepsilon1/\zeta1$, $\varepsilon2/\zeta1$ and $\varepsilon3/\zeta1$ heteromeric channels [9]. The order of the affinities for L-glutamate and glycine is $\varepsilon4/\zeta1 > \varepsilon3/\zeta1 > \varepsilon2/\zeta1 > \varepsilon1/\zeta1$ channels. The effects of APV and 7CK were tested at agonist concentrations as high as ~10 times the EC_{50} values (Fig.3B). The $\varepsilon4/\zeta1$ channel activity was reduced only by 31% in the presence of 100 μM APV and by 34% in the presence of 3 μM 7CK. The extents of inhibition are smaller than those observed for the other ε/ζ heteromeric channels under similar conditions (69–98% and 36–75%, respectively) [9]. The sensitivity to APV is in the order $\varepsilon1/\zeta1 > \varepsilon2/\zeta1 > \varepsilon3/\zeta1 > \varepsilon4/\zeta1$ channels, whereas that to 7CK is in the order $\varepsilon3/\zeta1 > \varepsilon2/\zeta1 > \varepsilon1/\zeta1 \approx \varepsilon4/\zeta1$ channels. The $\varepsilon4/\zeta1$ heteromeric channel is thus characterized by high affinity for agonist and low sensitivity to competitive antagonist.

Our previous studies suggest that the molecular diversity of the ε subunit family underlies the functional heterogeneity of the NMDA receptor channel [9]. The identification and functional characterization of the $\varepsilon4$ subunit reveal further diversity of the NMDA receptor channel.

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