A Single Tryptophan on M2 of Glutamate Receptor Channels Confers High Permeability to Divalent Cations

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ABSTRACT Ionotropic glutamate receptors (iGluRs) of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate subtype display lower permeability to Ca^{2+} than the *N*-methyl-D-aspartate (NMDA) subtype. The well-documented N/Q/R site on the M2 transmembrane segment (M2) is an important determinant of the distinct Ca^{2+} permeability exhibited by members of the non-NMDA receptor subfamily. This site, however, does not completely account for the different permeation properties displayed by non-NMDA and NMDA receptors, suggesting the involvement of other molecular determinants. We have identified additional molecular elements on M2 of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate receptor GluR1 that specify its permeation properties. Higher permeability to divalent over monovalent cations is conferred on GluR1 by a tryptophan at position 577, whereas blockade by external divalent cations is imparted by an asparagine at position 582. Hence, the permeation properties of ionotropic glutamate receptors appear to be primarily specified by two distinct determinants on M2, the well-known N/Q/R site and the newly identified L/W site. These findings substantiate the notion that M2 is a structural component of the pore lining.

INTRODUCTION

Ionotropic glutamate receptors (iGluRs) are neurotransmitter-activated ion channels that mediate excitatory synaptic transmission in the central nervous system (CNS) (Collingridge and Lester, 1989; Choi, 1992; Nakanishi, 1992). According to their pharmacological and physiological properties, this family of ligand-gated ion channels is classified in two major subtypes: *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (Collingridge and Lester, 1989; Choi, 1992; Nakanishi, 1992; Gasic and Hollmann, 1992; Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994). A hallmark of the NMDA receptor channel, in contrast to non-NMDA receptors, is its high Ca^{2+} permeability and sensitivity to blockade by extracellular Mg^{2+} and other divalent cations (Mayer and Westbrook, 1987; Iino et al., 1990; Gilbertson et al., 1991; Jahr and Stevens, 1993; Zarei and Dani, 1994). The high Ca^{2+} permeability makes the NMDA receptor a pivotal entity for both the function and dysfunction of the CNS (Collingridge and Lester, 1989; Choi, 1992; Nakanishi, 1992). Accordingly, NMDA receptors are considered key players in Ca^{2+}-dependent synaptic plasticity processes such as long-term potentiation, a phenomenon associated with learning and memory (Collingridge and Lester, 1989; Jessell and Kandel, 1993; Stevens, 1993). Likewise, iGluRs are implicated in neurotoxic events (Choi and Rothmann, 1990; Olney, 1990). Indeed, glutamate-mediated overstimulation of iGluRs, particularly the NMDA receptor channel, triggers a massive influx of Ca^{2+} into neurons, leading to neuronal cell death (Choi and Rothmann, 1990).

Elucidation of the molecular determinants that specify the distinctive permeation properties of iGluRs is a target of intense research. Availability of the primary structure of the different receptor subunits has led to the identification of molecular components involved in modulating the permeation properties of this receptor channel (Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994). An asparagine residue (N598 on NMDA receptor subunit 1 (NMDAR1), Fig. 1) at the C-terminal half of M2 regulates both its ionic permeability and pore blockade properties (Burnashev et al., 1992b; Mori et al., 1992; Sakurada et al., 1993). The corresponding residue on non-NMDA receptors, a glutamine or arginine, contributes to their permeation properties (Verdoorn et al., 1991; Hume et al., 1991; Mishina et al., 1991; Dingledine et al., 1992; Curutchet et al., 1992; Jonas and Burnashev, 1995). Furthermore, the amino acid at this critical position, known as the N/Q/R site, is controlled by RNA editing (Sommer et al., 1991; Burnashev et al., 1992a; Jonas and Burnashev, 1995). The N/Q/R site, however, is not sufficient to account for the different ionic permeability and pore blockade properties displayed by NMDA and non-NMDA receptors. A case in point is the modest increase in the Ca^{2+} and Ba^{2+} permeabilities caused by replacement of the glutamine in non-NMDA receptors, such as GluR1 or GluR3, by asparagine (Burnashev et al., 1992a; Dingledine et al., 1992); yet these permeabilities are far lower than that characteristic of the NMDA receptor (Mayer and Westbrook, 1987; Iino et al., 1990; Jahr and Stevens, 1993; Zarei and Dani, 1994). Taken together, these observations suggest that other molecular determinants may be involved in the specification of the distinct permeation properties of the ionotropic glutamate receptor gene family.

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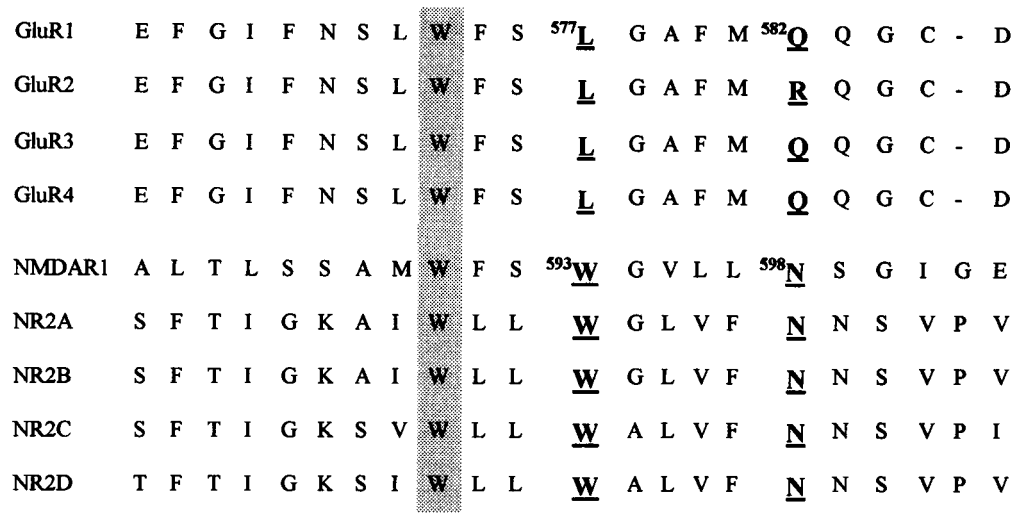

FIGURE 1 Sequence alignment of the M2 transmembrane segments of the AMPA/KA receptor subunits GluR1–4 and all of the NMDA receptor subunits (Hollmann and Heinemann, 1994). Shadowed area denotes amino acid conservation. Residues in bold and underlined indicate conservation among the AMPA or the NMDA receptor subunits. For GluR2 the edited version is displayed.

In this paper, we report the identification of additional determinants. Our experimental approach involves the stepwise mutation of GluR1 to mimic the amino acid residues of NMDAR1 at positions presumably involved in specifying its permeation properties. The restructured receptors are functionally expressed and assayed as homooligomers in *Xenopus* oocytes. This strategy has been used to identify pore-lining residues on glycine receptors (Galzi et al., 1992), the scorpion toxin receptor of a potassium channel (Gross et al., 1994), and the phencyclidine (PCP) and dizolcipine (MK-801) binding sites on the NMDA receptor (Ferrer-Montiel et al., 1995b).

Fig. 1 shows an alignment of amino acid sequences of M2 for the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate (AMPA/KA) receptor subunits GluR1–4, and for all of the NMDA receptor subunits cloned thus far. (Hollmann and Heinemann, 1994). We focus on M2 because it is thought of as a component of the channel lining (Mori et al., 1992; Dingledine et al., 1992; Burnashev et al., 1992a,b; Sakurada et al., 1993; Montal, 1995; Ferrer-Montiel et al., 1995b). Two residues that determine pore blockade properties, W593 and N598 on M2 of the NMDAR1, are absolutely conserved among NMDA receptor subunits (Burnashev et al., 1992b; Sakurada et al., 1993; Ferrer-Montiel et al., 1995b). The corresponding residues on M2 of GluR1 are L577 and Q582. Note that L577 is conserved among the AMPA/KA receptor subunits GluR1–4. As described in this paper, the main consequence of our analysis is that high Ca^{2+} permeability and channel block by extracellular divalent cations are primarily determined by two different amino acids on M2 of GluR1: an aromatic residue at position 577 confers on GluR1 higher permeability to Ca^{2+} and Ba^{2+} over Na^{+}, whereas an asparagine at position 582 imparts sensitivity to block by extracellular divalent cations. A preliminary account of this work was presented elsewhere (Ferrer-Montiel et al., 1995a).

MATERIALS AND METHODS

Site-directed mutagenesis, cRNA preparation, and microinjection into *Xenopus* oocytes

GluR1 is a cDNA clone encoding a functional AMPA/KA receptor from human brain (Sun et al., 1992). NR1 (Planells-Cases et al., 1994) and NR2A (Le Bourdelles et al., 1994) (kindly provided by Dr. Paul Whiting) are cDNA clones encoding two subunits of an NMDA receptor from human brain. Site-directed mutagenesis was carried out by using single-stranded DNA (Kunkel et al., 1991) or by PCR as described (Ferrer-Montiel et al., 1995b). Mutant receptors were confirmed by DNA sequencing. Capped cRNA (Kreig and Melton, 1984) was synthesized from linearized cDNA, using the mMESSAGE mMACHINE from AMBION (Austin, Texas). For the single mutants, the number indicates the position of the residue in the protein sequence; the first letter is the natural amino acid in the wild-type protein, and the second is the residue that substitutes it. Double and triple mutants are denoted by their point mutations separated by a slash (/).

cRNA (0.4 mg/ml in diethylpyrocarbonate-treated water) was microinjected (V = 50 nl) into defolliculated oocytes (Stage V and VI) as described (Ferrer-Montiel and Montal, 1994). Oocytes were functionally assayed 3–6 days after cRNA injection. Recombinant NMDA receptors were obtained by coinjection of NR1 and NR2A subunits at a ratio of 1:3 (10 ng/oocyte).

Electrophysiological characterization of the GluR1 mutants

KA-evoked whole-cell currents were measured under voltage clamp (Turbo TEC O1C; NPI Electronics, Tamm, Germany) with a two-microelectrode voltage clamp (Ferrer-Montiel and Montal, 1994). Oocytes were transferred to the recording chamber ($V \approx 0.2$ ml) and were perfused (2–4 ml/min) with the appropriate Ringer's solution (Table 1) in the absence and/or presence of 0.5 mM KA as the GluR1 agonist. *I-V* characteristics were recorded using a ramp protocol (pClamp 5.5; Axon Instruments, Foster City, CA): oocytes were depolarized from −80 mV to 40 mV in 2 s (60 mV/s). Leak currents were measured in the absence of KA in the external bath medium and subtracted from the ionic current recorded in the presence of the ligand. The junction potential between the ground electrode and bath consequent to changing the extracellular ionic conditions from 10

TABLE 1 Ringer's solutions used for studying the ion permeation properties

	[NaCl] (mM)	[$BaCl_2$] (mM)	[$CaCl_2$] (mM)	[NMG] (mM)
125 Na/2 Ba	125	1.8	0	0
90 Na/2 Ba	90	1.8	0	25
50 Na/2 Ba	50	1.8	0	65
10 Na/2 Ba	10	1.8	0	115
10 Na/5 Ba	10	5	0	110
10 Na/10 Ba	10	10	0	100
10 Na/20 Ba	10	18	0	80
10 Na/2 Ca	10	0	1.8	115
10 Na/10 Ca	10	0	10	100
10 Na/20 Ca	10	0	18	80
70 Na/0.2 Ba	70	0.18	0	40
70 Na/2 Ba	70	1.8	0	36
70 Na/10 Ba	70	10	0	20
70 Na/20 Ba	70	18	0	0
70 Na/0.2 Ca	70	0	0.18	40
70 Na/2 Ca	70	0	1.8	36
70 Na/20 Ca	70	0	18	0

All Ringer's solutions contained 10 mM TES (pH 7.4) and 2.8 mM KCl. Normal Ba^{2+}-Ringer solution is the same as 125 Na/2 Ba. NMG denotes *N*-methylglucamine.

Na/2 Ba (Ca) to 10 Na/20 Ba (Ca) was 2 mV. The reversal potentials reported were corrected accordingly.

To prevent or minimize the activation of the Ca^{2+}-activated, voltage-dependent Cl^- channel, KA was applied for <10 s, and all *I-V* curves were recorded from oocytes microinjected (V = 50 nl) with 120 mM EGTA-KOH, pH ~7.0, giving a final intracellular concentration of ~10 mM. The absence of Cl^- current contamination was confirmed by the EGTA inhibition of outward currents at positive membrane potentials in rectifying GluR1 channels, and by the blockade of the KA-evoked ionic current produced by increases in $[Ba^{2+}]_o$ and $[Ca^{2+}]_o$.

Inhibition constants were determined from dose-response curves (Sun et al., 1992). Oocytes were challenged with 5-s, 0.5-mM KA pulses in the appropriate Ringer's solution supplemented with increasing concentrations of the channel blockers. Dose-response curves were fitted to a Michaelis-Menten binding isotherm:

$$I = I_{max}/[1 + (\text{BLOCKER})^{n_h}/IC_{50}],$$

where IC_{50} is the inhibition constant and denotes the concentration of blocker needed to inhibit half of the maximum response (I_{max}) recorded in its absence, and n_h is the steepness of the inhibition curve.

Additional details concerning recording and procedures are as described elsewhere (Ferrer-Montiel and Montal, 1994).

Calculation of the relative ionic permeabilities using the GHK equation

To determine the relative ionic permeabilities of K^+, Ba^{2+}, and Ca^{2+} with respect to Na^+, we applied the constant field approximation using the GHK equation (Lewis, 1979; Mayer and Westbrook, 1987). Because of the high intracellular EGTA concentration (~10 mM) used in these studies, the contribution of Cl^- permeability to the reversal potentials was considered negligible. The GHK equation, modified to include the contribution of the permeability to divalent cations, is (Lewis, 1979; Mayer and Westbrook, 1987; Ferrer-Montiel and Montal, 1993)

$$V_r = \frac{RT}{F}\ln\frac{[Na^+]_o + \frac{P_{K^+}}{P_{Na^+}}[K^+]_o + 4\frac{P'_{Ba^{2+}}}{P_{Na^+}}[Ba^{2+}]_o}{[Na^+]_i + \frac{P_{K^+}}{P_{Na^+}}[K^+]_i + 4\frac{P'_{Ba^{2+}}}{P_{Na^+}}[Ba^{2+}]_i \exp\left(\frac{FV_r}{RT}\right)},$$

with

$$P'_{Ba^{2+}} = \frac{P_{Ba^{2+}}}{1+\exp(FV_r/RT)},$$

where P_{K^+}/P_{Na^+} and $P_{Ba^{2+}}/P_{Na^+}$ denote the relative permeabilities of K^+ and Ba^{2+} (or Ca^{2+}: $P_{Ca^{2+}}/P_{Na^+}$) with respect to Na^+; $[X]_i$ and $[X]_o$ refer to the intracellular and extracellular activities of the permeant ions (Na^+, K^+, and Ba^{2+}). RT/F is 25.3 mV at 20°C; $[Na^+]_i$ = 10 mM, $[K^+]_i$ = 120 mM, $[Ba^{2+}]_i$ = 0, $[K^+]_o$ = 2.8 mM. The intracellular ion concentration of 130 mM produced the minimum χ^2 in the fitting of the experimental data to the GHK equation. Activity coefficients (γ_i) for divalent cations were taken from Mayer and Westbrook (1987) and were assumed to vary linearly with the ionic strength of the extracellular ionic solutions used:

$$\gamma_{Ba} = (\gamma_{BaCl})^2; \quad \gamma_{Ca} = (\gamma_{CaCl})^2.$$

Accordingly, $\gamma_{Ba^{2+}}$ = 0.324 for $[Ba^{2+}]$ = 1.8 mM, and $\gamma_{Ba^{2+}}$ = 0.295 for $[Ba^{2+}]$ = 18 mM. The ionic activity was calculated as $\gamma \times$ molarity. For Na^+ and K^+, γ = 0.78 (Mayer and Westbrook, 1987). Reversal potentials were plotted as a function of the extracellular ionic activity and fitted to the GHK equation with a nonlinear least-squares regression algorithm using MicroCal ORIGIN, version 2.8 (Microcal, Amherst, MA) and MATLAB (MathWorks, Natick, MA) (Ferrer-Montiel and Montal, 1996). The goodness of fit was inferred from the χ^2 test (Colquhoun, 1971).

RESULTS AND DISCUSSION

Residues at positions 577 and 582 on M2 of GluR1 modulate the permeation properties

Homomeric GluR1 channels display an inwardly rectifying *I-V*, as illustrated in Fig. 2 *A* (*top left*). The KA-evoked ionic current is largely carried by Na^+, as evidenced by the dependence of both the reversal potential (V_r) and the current amplitude on the Na^+ concentration in the external bath medium ($[Na^+]_o$). A ninefold increment in $[Na^+]_o$ produced a ~35-mV shift in V_r toward more depolarizing potentials (Table 2) and increased the amplitude of the KA-elicited ionic current (Fig. 2 *A*, *top left*). Similarly, Ba^{2+} and Ca^{2+} permeate through GluR1. As shown in Fig. 2 *B* (*top left*), recorded in 10 mM $[Na^+]_o$, V_r was displaced ~24 mV to positive potentials when the extracellular ionic concentrations of Ba^{2+} and Ca^{2+} ($[Ba^{2+}]_o$, $[Ca^{2+}]_o$) were increased 10-fold (Table 2). These findings indicate that GluR1 is permeable to both monovalent and divalent cations.

Mutation of L577 to tryptophan did not affect the *I-V* characteristics or the Na^+ permeability (Fig. 2 *A*, *top right*; Table 2), yet it altered the permeability to divalent cations (Fig. 2 *B*, *top right*; Table 2). As shown, the amplitude of the KA-activated ionic current increased with $[Ba^{2+}]_o$. Furthermore, a 10-fold increase in $[Ba^{2+}]_o$ or $[Ca^{2+}]_o$ shifted V_r ~33 mV toward more depolarizing potentials (Table 2). This displacement of V_r was ~10 mV larger than that observed for GluR1, suggesting that the L577W mutant receptor exhibits higher permeability for divalent cations than does GluR1. At variance with the L577W single mutant, receptors incorporating an asparagine at position 582, Q582N, displayed a linear *I-V* (Fig. 2 *A*, *bottom left*), in accord with Dingledine et al. (1992), and unaffected ionic

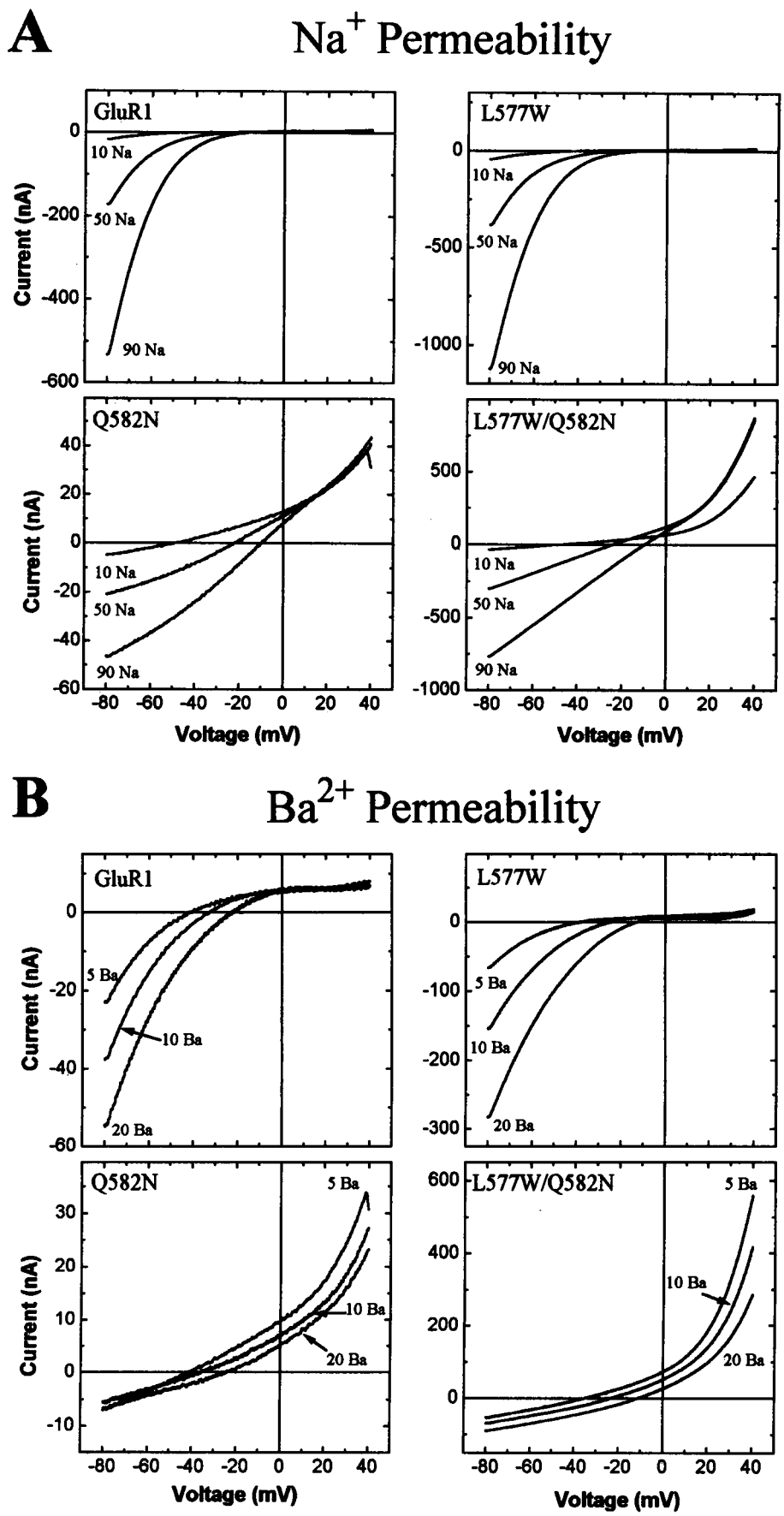

FIGURE 2 Mutations of L577 and Q582 on M2 regulate the permeation properties of GluR1. (*A*) *I-V* characteristics of ionic currents elicited by 0.5 mM KA at different $[Na^+]_o$. 10 Na, 50 Na, and 90 Na denote Ba^{2+}-Ringer's solution containing 10, 50, and 90 mM Na^+, respectively. (*B*) *I-V* curves elicited by 0.5 mM KA in Ringer's solution containing 5 mM (5 Ba), 10 mM (10 Ba), and 18 mM (20 Ba) Ba^{2+}. The $[Na^+]_o$ was 10 mM. Each trace is representative of at least five oocytes. Oocytes were held at −80 mV in the appropriate buffer and depolarized to 40 mV in 2 s (60 mV/s) using a ramp protocol. Leak currents were obtained in the absence of KA and substracted from the KA-evoked ionic currents.

TABLE 2 Reversal potentials of GluR1 mutants

Species	V_r (mV)					*I-V*
	10 Na/2 Ba	90 Na/2 Ba	10 Na/20 Ba	10 Na/2 Ca	10 Na/20 Ca	
GluR1	−48 ± 5	−13 ± 3	−24 ± 3	−50 ± 3	−25 ± 3	Inwardly rectifying
L577W	−42 ± 2	−15 ± 3	−9 ± 2	−48 ± 3	−14 ± 2	Inwardly rectifying
A579V	−52 ± 3	−12 ± 3	−26 ± 2	−50 ± 3	−24 ± 3	Inwardly rectifying
Q582N	−49 ± 2	−10 ± 2	−26 ± 3	−46 ± 4	−24 ± 4	Linear
L577W/Q582N	−45 ± 3	−10 ± 1	−12 ± 1.4	−55 ± 3	−21 ± 1	Linear
L577W/Q582T	−46 ± 1	−16 ± 3	−13 ± 1.6	−52 ± 2	−14 ± 2	Inwardly rectifying
M581L/Q582N	−52 ± 4	−11 ± 2	−40 ± 4	−52 ± 1	−29 ± 4	Linear
NMDAR	−32 ± 2	—	−2.5 ± 2.3	−36 ± 2	−3 ± 2	Linear

Reversal potentials (V_r) were obtained from *I-V* relationships as the voltage at which the KA-activated ionic current was zero. Ionic currents were elicited by 0.5 mM KA. Ramps were evoked from −80 mV to 40 mV in 2 s as described in Fig. 2. External ionic compositions were 10 mM Na^+, 1.8 mM Ba^{2+} (10 Na/2 Ba); 90 mM Na^+, 1.8 mM Ba^{2+} (90 Na/2 Ba); 10 mM Na^+, 18 mM Ba^{2+} (10 Na/20 Ba); 10 mM Na^+, 1.8 mM Ca^{2+} (10 Na/2 Ca); 10 mM Na^+, 18 mM Ca^{2+} (10 Na/20 Ca). Data are given as mean ± SEM, $n \geq 6$. GluR1 and mutant receptors exhibited an EC_{50} for KA of 30–40 μM. Ionic currents evoked by 0.5 mM KA at −80 mV for GluR1 and mutants were 0.1–1.0 μA for GluR1, A579V, L577W/Q582N, M581L/Q582N; 0.01–0.2 μA for Q582N; 0.2–2.0 μA for L577W and L577W/Q582T. NMDA receptors (NR1:NR2A, 1:3), activated with 100 μM L-glutamate and 20 μM glycine, produced ionic currents of 0.2–2.0 μA. *I-V* characteristics are referred to as linear or inwardly rectifying, as determined under physiological conditions in normal Ba^{2+}-Ringer solution (Ferrer-Montiel et al., 1995b).

permeabilities (Fig. 2, *A* and *B, bottom left*; Table 2). Replacement of A579 by valine, or the double mutation M581L/Q582N, produced minor effects on the *I-V* characteristics and ionic permeability (Table 2).

Simultaneous mutation of L577 and Q582, L577W/Q582N, produced receptor channels with a linear *I-V* relationship, and monovalent cation permeability similar to that of both single mutants (Fig. 2 *A, bottom right*; Table 2). The double mutant, however, was highly permeable to Ba^{2+} and Ca^{2+} (Fig. 2 *B, bottom right*; Table 2); $[Ba^{2+}]_o$ or $[Ca^{2+}]_o$ increased 10 times, and V_r was displaced 33 mV toward depolarizing potentials, as observed with the L577W single mutant. Note that removal of $[Na^+]_o$ resulted in outwardly rectifying currents arising from K^+ efflux.

To further examine the functional role of the residue at position 582, we introduced a threonine or an aspartic acid. These two residues, with an spatial volume similar to that of asparagine (~95 $Å^3$), probe the contribution of hydroxyl or carboxyl groups at this position. Unexpectedly, the Q582T, Q582D, and L577W/Q582D mutant receptors did not express functional channels. In contrast, the double mutant L577W/Q582T produced receptors that display an inwardly rectifying *I-V* and high permeability to divalent cations, similar to that exhibited by L577W single and L577W/Q582N double mutants (Table 2). These results, therefore, suggest that the residue at position 577 on M2 of GluR1 may be an additional molecular determinant of the divalent cation permeability exhibited by iGluRs.

A tryptophan at position 577 on M2 of GluR1 increases the permeability of divalent over monovalent cations

To assess whether the residues implanted on M2 of GluR1 specify the ionic selectivity, we determined the permeabilities to monovalent and divalent cations. Permeabilities were calculated using the Goldman-Hodgkin-Katz (GHK) equation modified to include the contribution of divalent cations (Fig. 3 *A*) (Lewis, 1979; Mayer and Westbrook, 1987; Ferrer-Montiel and Montal, 1993). Reversal potentials were measured in the presence of ~10 mM EGTA in the intracellular medium to minimize the contribution of the Cl^- permeability, which is primarily carried by the Ca^{2+}-activated, voltage-dependent Cl^- channel (Miledi and Parker, 1984). The relative permeability of GluR1 channels to monovalent cations, P_{K^+}/P_{Na^+}, was 1.0, implying equivalent selectivity of the channel for K^+ and Na^+. The permeability for divalent cations was 1.9. As illustrated in Fig. 3 *B*, the P_{K^+}/P_{Na^+} was modestly increased by mutating L577 to tryptophan on M2; P_{K^+}/P_{Na^+} = 1.3–1.5.

Incorporation of a tryptophan at position 577, however, markedly increased both $P_{Ba^{2+}}/P_{Na^+}$ and $P_{Ca^{2+}}/P_{Na^+}$. As shown in Fig. 3 *B*, L577W single mutant and L577W/Q582N and L577W/Q582T double mutants exhibited $P_{Ba^{2+}}$ and $P_{Ca^{2+}}$ compared to P_{Na^+} of 4–6. Mutation of Q582 to asparagine did not augment the change ($P_{Ba^{2+}}/P_{Na^+}$ = 1.9). Hence, these findings indicate that the residue at position 577 on M2 of GluR1 is a structural determinant of the divalent cation selectivity. An aromatic residue at this position produces homomeric GluR1 receptors with divalent cation permeability closer to that characteristic of the NMDA receptor, $P_{Ba^{2+}}/P_{Na^+}$ and $P_{Ca^{2+}}/P_{Na^+}$ = 7–8 (Fig. 3, *A* and *B*). Interestingly, homomeric GluR6 receptors of the unedited version, which have a valine at position 616 (corresponding to 577 in GluR1), exhibit lower permeability to divalent cations ($P_{Ca^{2+}}/P_{Na^+}$ = 1.1) than homomeric GluR1 receptors (Egebjerg and Heinemann, 1993). Taken together, these observations suggest that the newly identified L/V/W site on M2 of iGluRs contributes specifically to the different permeation properties exhibited by the iGluR subtypes.

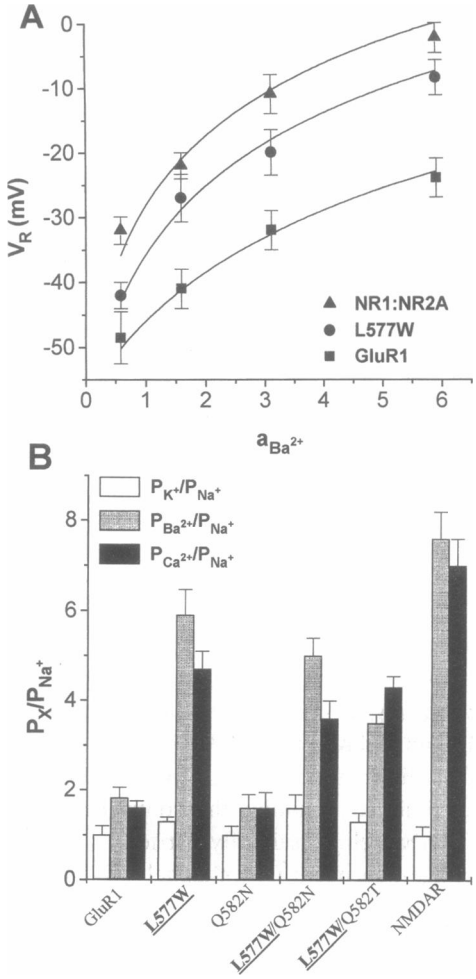

FIGURE 3 A tryptophan at position 577 on M2 confers on GluR1 high permeability to divalent over monovalent cations. (*A*) Reversal potentials for GluR1, L577W mutant, and recombinant NMDAR plotted as a function of the extracellular Ba^{2+} activity. Solid lines depict the best fit to the GHK equation using a nonlinear square algorithm. The goodness of the fit was assessed from the γ^2 test. Relative permeabilities of K^+ and Ba^{2+} to Na^+ were 1.0 ± 0.1 and 1.9 ± 0.3 for GluR1; 1.3 ± 0.2 and 6.0 ± 0.3 for L577W; and 1.0 ± 0.1 and 7.9 ± 0.2 for NMDAR. (*B*) Relative permeabilities of K^+ (P_{K^+}/P_{Na^+}), Ba^{2+} ($P_{Ba^{2+}}/P_{Na^+}$), and Ca^{2+} ($P_{Ca^{2+}}/P_{Na^+}$) with respect to Na^+. Data are given as mean ± SEM, with $n \geq 6$.

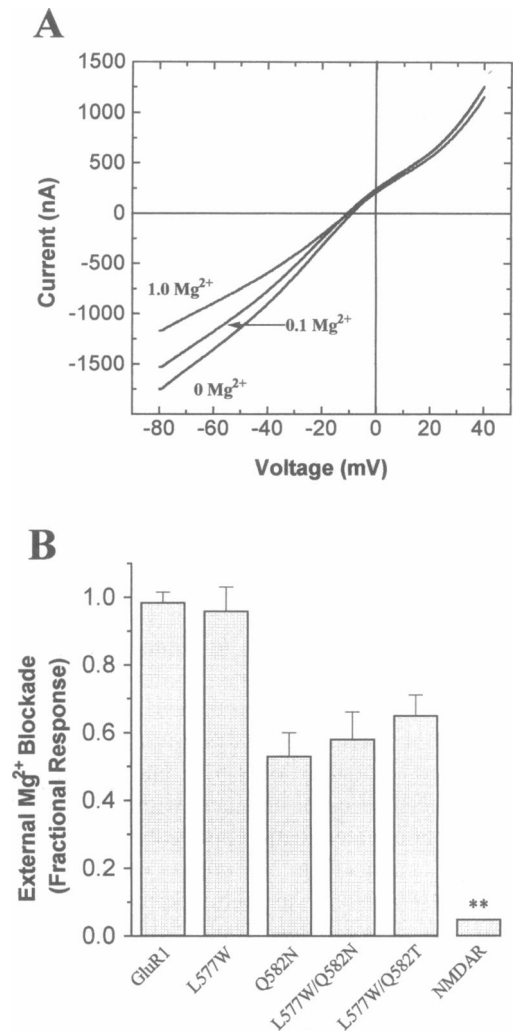

FIGURE 4 GluR1 mutant receptors are blocked by extracellular Mg^{2+}. (*A*) *I-V* curves from L577W/Q582N double mutant elicited by 0.5 mM KA in the absence and presence of 0.1 and 1 mM Mg^{2+} in low Ba^{2+}-Ringer's solution (0.18 mM Ba^{2+}). Traces are representative of three oocytes. Similar results were obtained for Q582N and L577W/Q58T mutants (data not shown). Other conditions are as described in Fig. 2. (*B*) Fractional response denotes the KA-activated ionic current in low Ba^{2+}-Ringer's solution supplemented with 1 mM Mg^{2+} with respect to that elicited in its absence, at a holding potential of −80 mV. Ionic currents were elicited by 5-s pulses of 0.5 mM [KA]. Oocytes were equilibrated for 5 min in the corresponding external bath medium before the agonist pulse. Values are given as mean ± SEM with $n \geq 4$. **, For the NMDA receptor, Mg^{2+} values are from Iino et al. (1990).

Mutant GluR1 channels are blocked by extracellular divalent cations

To further understand the role of residues L577 and Q582 in the permeation properties of homomeric GluR1, we investigated the sensitivity to blockade by extracellular divalent cations. As shown in Fig. 4 *A*, extracellular Mg^{2+} reduced the KA-evoked current of mutant GluR1 channels at negative potentials. To quantify the extent of channel block, the fractional response was determined as the ratio of the KA-evoked ionic current in the presence of 1 mM Mg^{2+} with respect to that elicited in its absence, at a holding potential of −80 mV (Fig. 4 *B*). As displayed, GluR1 channels were insensitive to 1 mM $[Mg^{2+}]_o$. In contrast, mutant GluR1 channels incorporating an asparagine or threonine at position 582 were blocked 40–50%. Mg^{2+} blockade was attenuated by increases in $[Ba^{2+}]_o$ or $[Ca^{2+}]_o$: a 10-fold increase in $[Ba^{2+}]_o$ drastically reduced $[Mg^{2+}]_o$ blockade (~80%; data not shown).

The restructured GluR1 receptors were also blocked by extracellular Ca^{2+} and Ba^{2+}, as illustrated in Fig. 5. Increasing $[Ba^{2+}]_o$ or $[Ca^{2+}]_o$ progressively diminished Na^+ current through the asparagine-containing GluR1 mutants at position 582 on M2. K^+ currents were also blocked by $[Ba^{2+}]_o$ (Fig. 2 *B, bottom*). Blockade was voltage indepen-

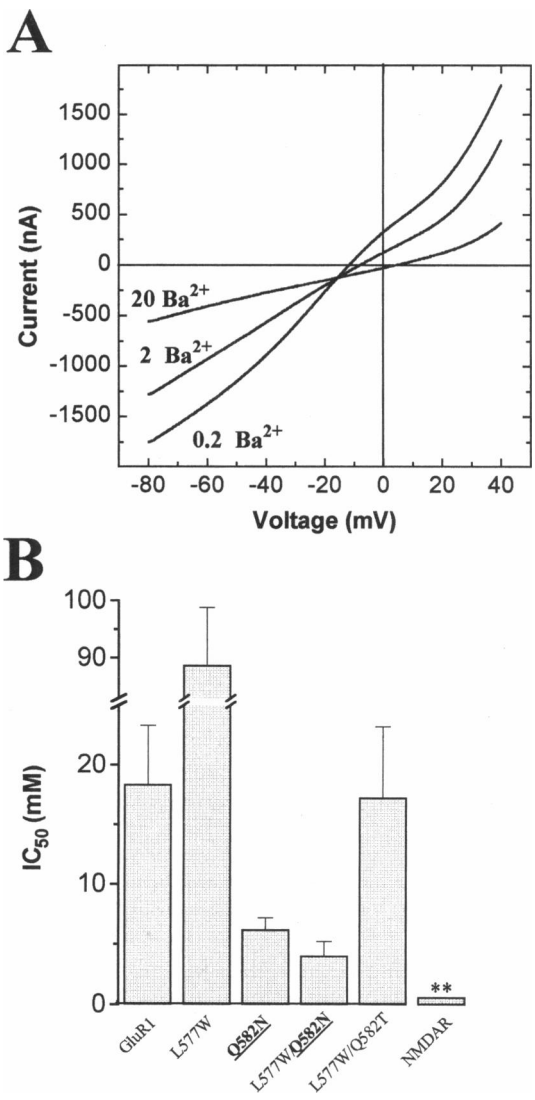

FIGURE 5 Extracellular Ba^{2+} blocks the Na^+ current through GluR1 mutant channels. (*A*) Voltage-independent block of Na^+ currents by $[Ba^{2+}]_o$. *I-V* curves from the L577W/Q582N double mutant obtained in modified Ba^{2+}-Ringer's solutions containing 0.18 mM (0.2 Ba^{2+}), 1.8 mM (2 Ba^{2+}), and 18 mM Ba^{2+} (20 Ba^{2+}); $[Na^+]_o$ was 70 mM. Traces are representative of five oocytes. Similar results were obtained for the Q582N mutant. Other conditions are as in Fig. 2. (*B*) IC_{50}, the inhibition constant, was obtained from the best fit of the experimental data to a Michaelis-Menten binding isotherm (Ferrer-Montiel and Montal, 1995b). Ionic currents were elicited by 5-s pulses of 0.5 mM KA. Oocytes were equilibrated for 5 min in the corresponding external bath medium before the agonist pulse. Values are given as mean ± SEM, with $n \geq 6$. **, For the NMDA receptor, IC_{50} values for Ba^{2+} blockade are from Raditsch and Witzemann (1994).

dent, as evidenced by similar inhibition at all voltages assayed, from −80 mV to 40 mV (Fig. 5 *A*).

To characterize the blockade of the KA-activated ionic current by extracellular divalent cations, we estimated the IC_{50}, i.e. $[Ba^{2+}]_o$ required to block half of the maximum response elicited by the agonist. As shown in Fig. 5 *B* for GluR1, $IC_{50} \approx 20$ mM. Strikingly, mutation of L577 to tryptophan decreased the sensitivity to block by $[Ba^{2+}]_o$ ($IC_{50} \approx 90$ mM). This finding is consistent with the remarkable increase in $P_{Ca}{}^{2+}$ and $P_{Ba}{}^{2+}$ displayed by this mutant receptor. In contrast, the Q582N single mutant exhibited higher sensitivity to $[Ba^{2+}]_o$ blockade, $IC_{50} \approx 4$ mM. Simultaneous mutation of L577 to tryptophan and Q582 to asparagine generated receptor channels with slightly higher affinity to $[Ba^{2+}]_o$ ($IC_{50} \approx 3$ mM; Fig. 5 *B*) and Ca^{2+} ($IC_{50} \approx 1.5$ mM; data not shown) than either single mutant. Surprisingly, replacements of Q582 by threonine decreased the sensitivity to $[Ba^{2+}]_o$ blockade: the IC_{50} for L577W/Q582T was ~20 mM, similar to that measured for GluR1 channels.

Together, these findings indicate that the residue at position 582 on M2 of GluR1 is a molecular determinant of the sensitivity to external divalent cation blockade. Both the chemical reactivity and size of the residue at this position specify the affinity for blockade. Noticeably, the restructured GluR1 receptors display lower affinity for Mg^{2+}, Ba^{2+}, and Ca^{2+} blockade than the NMDA receptor (Ascher and Nowak, 1988; Mori et al., 1992; Burnashev et al. 1992b; Sakurada et al., 1993; Mayer and Westbrook, 1987; Jahr and Stevens, 1993; Raditsch and Witzemann, 1994), suggesting that other residues and/or subunits may be involved in divalent cation binding. Indeed, on heteromeric NMDA receptors, mutation of N598 to glutamine of NMDAR1 has minor effects on the Mg^{2+} sensitivity, whereas mutation of the corresponding residue on NR2A drastically reduces Mg^{2+} block (Burnashev et al., 1992b). In contrast, heteromeric NMDA receptors formed by NMDAR1 and NR2C are modestly sensitive to Mg^{2+} block (Mishina et al., 1991). Specific subunits, therefore, may account for the disparity in divalent cation sensitivity between heteromeric NMDA receptors (Fig. 1) and our results on homomeric GluR1 receptors.

Inferences about the relationship between the structure of M2 and its function as a pore liner of the glutamate receptor channels

Caution must be exercised when inferring protein structure from functional assays using site-directed mutagenesis. Point mutations on a protein may cause structural rearrangements, leading to a global change in the protein fold. Hence, the functional effects could arise from long-range structural changes rather than local perturbations. Our results, however, argue against a drastic change on the GluR1 fold as a result of the mutations (Ferrer-Montiel et al., 1995b): 1) the agonist sensitivity of all mutant channels was virtually identical; 2) the kinetics of the KA-evoked response was not altered; 3) the level of protein expressed was unaffected; 4) mutation of L577 modified primarily PCP binding and divalent cation permeability, independently of mutations of other residues on M2; and 5) substitutions of Q582 modulated the pore blockade properties and *I-V* characteristics, irrespective of the amino acid at position 577 on M2. Thus,

the functional effects reported here appear to result from specific local changes on protein structure introduced by site-selective mutagenesis.

A question, therefore, arises: Are our functional observations compatible with a particular structural model for the ionic pore? The key finding that L577 and Q582 on M2 of GluR1 define distinct permeation properties suggests that these residues are exposed to the channel lumen and interact with the permeant ion. If M2 were a regular α-helix spanning the lipid bilayer, residues L577 and Q582 would face opposite sides of the helix, a spatial arrangement inconsistent with their role as molecular determinants of selectivity and block. It is noteworthy that Kuner et al. (1995) probed the secondary structure of M2 of NMDA receptor channels using cysteine mutants and concluded that the results were inconsitent with a simple α-helical structure. Structural data for the acetylcholine receptor (AChR) at 9 Å resolution reveal the presence of a rod of density, interpreted as an α-helix, in the membrane domain of each subunit (Unwin, 1993a,b, 1995). This α-helix, presumably M2, is bent in the middle at the level of leucine-251 (Unwin, 1993b). Using the cysteine-substitution approach, Akabas et al. (1994) inferred that the AChR M2 is an interrupted α-helix. In analogy to the AChR, an irregular helical structure at the level of L577 on M2 of GluR1 could expose this residue and Q582 to the channel lumen. Interestingly, the amino acid adjacent to L577 is glycine (Fig. 1), a residue well known for its propensity to break α-helical conformations. Thus, a bent or shortly extended helix may be compatible with the specific effects determined by L577 and Q582. A kink or extension near position 577 would provide flexibility to M2 which, in the open conformation, presumably projects this residue side chain into the pore lumen, as postulated for the AChR (Unwin, 1995). An aromatic residue at this position may increase the permeability to divalent over monovalent cations through a mechanism involving π-cation interactions, as proposed for voltage-gated K^+ channels (Kumpf and Dougherty, 1993).

Based on an *N*-glycosylation site tagging approach, a topology for GluR1 involving only three transmembrane segments was proposed (Hollmann et al., 1994). In this model, M2 does not span the entire lipid bilayer, but rather it loops around near the middle, forming a β-hairpin-like structure, resembling that proposed for the H5 segment of voltage-gated ion channels (cf. McKinnon, 1995; Wo and Oswald, 1995). Similarly, Wo and Oswald (1994) and Bennett and Dingledine (1995) reported evidence in favor of a three-transmembrane segment model for kainate-binding proteins and a glutamate receptor channel. This view is consistent with the occurrence of glycosylation sites in the loop connecting M3 and M4 (Roche et al., 1994), a segment that appears to determine aspects of ligand binding on glutamate receptors (Stern-Bach et al., 1994). According to this topology, residue 577 in GluR1 would be located near the center of the presumed loop, a key position for determining its ionic permeability (Sutcliffe et al., 1996). Q582 would be located near the cytoplasmic face of the pore, a position consistent with the voltage-dependent block by extracellular Mg^{2+} of NMDA receptors (Asher and Nowak, 1988). A three-transmembrane-segment topology is compatible with observations that assign a role to M1 (Köhler et al., 1993) and M3 (Ferrer-Montiel et al., 1995b) in regulating pore properties. A deep location of Q582 in the ionic pore of GluR1, however, appears inconsistent with data ascribing an important role to this residue in the design of the binding pocket for Joro spider toxin (Blaschke et al., 1993), argiotoxin (Herlitze et al., 1993), and both PCP and MK-801 (Ferrer-Montiel et al., 1995b). Moreover, phosphorylation studies on the NMDA receptor constrain part of the loop connecting M3 and M4 to the intracellular domain, in disaccord with a three-transmembrane-segment model (Raymond et al., 1993; Wang et al., 1993; Nakazawa et al., 1995).

The two distinct topological models of glutamate-gated ion channels postulate that residues L577 and Q582 are exposed to the channel lumen, in accord with our findings. However, our data do not favor one or the other, yet contribute valuable information for the current ongoing refinements of receptor structure models. It is noteworthy that, irrespective of their topology, iGluRs appear to be pentameric assemblies (Béhé et al., 1995; Ferrer-Montiel and Montal, 1996), suggesting a conserved subunit stoichiometry of the ligand-gated ion channel superfamily.

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