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A Regulatory Domain (R1–R2) in the Amino Terminus of the *N*-Methyl-D-Aspartate Receptor: Effects of Spermine, Protons, and Ifenprodil, and Structural Similarity to Bacterial Leucine/Isoleucine/Valine Binding Protein

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ABSTRACT

There are complex interactions between spermine, protons, and ifenprodil at *N*-methyl-D-aspartate receptors. Spermine stimulation may involve relief of proton inhibition, whereas ifenprodil inhibition may involve an increase in proton inhibition. We studied mutations at acidic residues in the NR1 subunit using voltage-clamp recording of NR1/NR2B receptors expressed in *Xenopus* oocytes. Mutations at residues near the site of the exon-5 insert, including E181 and E185, reduced spermine stimulation and proton inhibition. Mutation NR1(D130N) reduced sensitivity to ifenprodil by more than 500-fold, but had little effect on sensitivity to spermine and pH. Mutations at six other residues in this region of the NR1 subunit reduced the potency and, in some cases, the maximum effect of ifenprodil. These mutants did not affect sensitivity to pH, glutamate, glycine, or other hallmark properties of *N*-methyl-D-aspartate channels such as Mg^{2+} block and Ba^{2+} permeability. Residues in this region presumably form part of the ifenprodil-binding site. To model this region of NR1 we compared the predicted secondary structure of NR1 (residues 19–400) with the known structures of 1,400 proteins. This region of NR1 is most similar to bacterial leucine/isoleucine/valine binding protein, a globular amino acid binding protein containing two lobes, similar to the downstream S1–S2 region of glutamate receptors. We propose that the tertiary structure of NR1(22–375) is similar to leucine/isoleucine/valine binding protein, containing two "regulatory" domains, which we term R1 and R2. This region, which contains the binding sites for spermine and ifenprodil, may influence the downstream S1 and S2 domains that constitute the glycine binding pocket.

N-methyl-D-aspartate (NMDA) receptors are modulated by a variety of endogenous and exogenous ligands, including polyamines such as spermine, and by protons. Spermine has complex effects on NMDA receptors, including two forms of stimulation and a voltage-dependent block (Williams, 1997). One of the best characterized effects of spermine is "glycine-independent" stimulation, seen in the presence of saturating concentrations of glutamate and glycine. At recombinant NMDA receptors, this form of stimulation is seen only at receptors containing splice variants of NR1 that lack the exon-5 insert, expressed alone or together with the NR2B subunit (Durand et al., 1993; Williams et al., 1994). NMDA receptors are inhibited by protons with an IC_{50} of pH 7.3 to 7.5. Thus, the receptors are tonically inhibited by about 50% at physiologic pH (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Traynelis et al., 1995). Spermine stimulation, which is pH sensitive, may involve relief of proton inhibition when responses are measured at physiologic pH (Traynelis et al., 1995). In support of this idea, a number of point mutations in the NR1 subunit that reduce spermine stimulation also reduce proton inhibition (Williams et al., 1995; Kashiwagi et al., 1996a, 1997; Traynelis et al., 1998). The effects of spermine and protons are both reduced by NR1 variants containing the exon-5 insert, and this insert may itself function as a spermine-like moiety and/or may shield the proton sensor (Zheng et al., 1994; Traynelis et al., 1995).

Ifenprodil is a novel NMDA antagonist that selectively

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; LIVBP, leucine/isoleucine/valine binding protein; QBP, glutamine binding protein; LAOBP, lysine/arginine/ornithine binding protein.

inhibits NR1/NR2 receptors containing the NR2B subunit (Williams, 1993; Williams et al., 1993). Ifenprodil does not act as a channel blocker nor as a competitive antagonist at the glutamate or glycine sites (Reynolds and Miller, 1989; Legendre and Westbrook, 1991; Williams, 1993). Furthermore, ifenprodil inhibits NMDA responses by a maximum of only 80 to 90% and it may act to allosterically inhibit channel opening (Legendre and Westbrook, 1991; Williams, 1993). It was suggested that ifenprodil is an antagonist at the spermine site (Carter et al., 1990), but ifenprodil inhibition is seen in the absence of extracellular spermine (Reynolds and Miller, 1989; Legendre and Westbrook, 1991; Williams, 1993) and recent evidence suggests that spermine and ifenprodil act at discrete sites with an allosteric interaction (Kew and Kemp, 1998). Ifenprodil has become widely used as a tool to study subtypes of NMDA receptors. In addition to its subtype-selectivity, an unusual feature of ifenprodil is a form of "activity-dependence", with block being dependent on agonist concentration. An increase in both the potency of ifenprodil and the maximal inhibition is seen with higher concentrations of NMDA or glutamate, and ifenprodil has a higher affinity for the agonist-bound and desensitized states of the receptor than for the closed or unbound states (Kew et al., 1996). Two other macroscopic effects of ifenprodil have been described. One is a small decrease in the affinity for glycine (Legendre and Westbrook, 1991; Williams, 1993) and the other is an interaction with proton inhibition (Pahk and Williams, 1997; Mott et al., 1998). Ifenprodil inhibition is pH-sensitive, with a larger inhibition at more acidic pH (Pahk and Williams, 1997). Furthermore, ifenprodil can apparently increase tonic proton inhibition and it has been suggested that this mechanism accounts for ifenprodil inhibition at NR1/NR2B receptors (Mott et al., 1998).

The sites of action of spermine, protons, and ifenprodil on NMDA receptors remain largely unknown. Residues in the extracellular amino terminal domain, the M3-M4 loop, and the M2 pore-forming region of the NR1 subunit have been found to influence sensitivity to spermine and pH (Sullivan et al., 1994; Williams et al., 1995; Kashiwagi et al., 1996a, 1997). In this paper we report that a cluster of acidic residues located near the exon-5 splice site influence sensitivity to spermine and pH. We have also identified seven residues in the proximal part of the amino terminus of NR1 that appear to form part of the ifenprodil binding site. A model is proposed based on the predicted secondary structure of the amino terminus NR1 and its similarity to leucine/isoleucine/valine binding protein (LIVBP).

Materials and Methods

NMDA Clones and Site-Directed Mutagenesis. The NR1 clone used in these studies is the NR1A variant (Moriyoshi et al., 1991), which lacks the 21-amino acid insert encoded by exon-5. This clone was a gift from Dr. S. Nakanishi (Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan). The NR2A and NR2B clones (Monyer et al., 1992) were gifts from Dr. P.H. Seeburg (Center for Molecular Biology, University of Heidelberg, Germany). Mutants were prepared by site-directed mutagenesis using the M13 phage system (Kunkel et al., 1987; Sayers et al., 1992). To prepare double or triple mutations in the same subunit, oligonucleotides for a second or third mutation were used with M13 fragments that already contained one or two mutations. Mutations were confirmed by DNA sequencing over approximately 100 nucleotides of the single strand M13 fragments containing the mutation. Because of the large number of mutants prepared in this study (a total of 127 mutants), a list of oligonucleotide primers used for mutagenesis has not been included but is available from the authors upon request.

Expression in Oocytes and Voltage-Clamp Recording. The preparation of capped cRNAs and the preparation, injection, and maintenance of oocytes were carried out as described previously (Williams, 1993, 1994; Williams et al., 1993). Oocytes were injected with NR1 plus NR2 cRNAs in a ratio of 1:5 (0.1–4 ng of NR1 plus 0.5–20 ng of NR2). Macroscopic currents were recorded with a two-electrode voltage clamp using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA) or an OC-725 amplifier (Warner Instruments, Hamden, CT) as described previously (Williams, 1993, 1994). Electrodes were filled with 3 M KCl and had resistances of 0.4 to 3 MΩ. Oocytes were continuously superfused with a saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES, pH 7.5) that contained $BaCl_2$ rather than $CaCl_2$ to minimize Ca^{2+}-activated Cl^- currents and in most experiments oocytes were injected with K^+-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA) (100 nl of 40 mM, pH 7.0–7.4) on the day of recording (Williams, 1993). To study the pH sensitivity of NMDA receptors, glutamate was applied in buffer at a given pH with a 30- to 60-s wash at that pH before and after application of glutamate. Concentration-response curves for glutamate and glycine were determined by using six to seven different concentrations of glutamate or glycine in the presence of a saturating concentration of the coagonist. Values for the agonist EC_{50} and for the pH IC_{50} were determined as described previously (Kashiwagi et al., 1996a, 1997). Voltage ramps (−100 to +60 mV over 4 s) were used to determine current-voltage profiles and to measure reversal potentials (V_{rev}) in extracellular Na^+-saline (saline solution; composition as above) and Ba^{2+}-saline (64 mM $BaCl_2$, 2 mM KCl, 10 mM HEPES, pH 7.5) as described previously (Williams et al., 1998). Leak currents were subtracted and the values of V_{rev} were corrected for small liquid junction potentials (+3 to +8 mV) measured in Ba^{2+}-saline versus Na^+-saline (Williams et al., 1998).

Results

Screening Mutations at Acidic Residues. Spermine is polycationic and we hypothesized that the amino groups of spermine may interact with acidic residues on NMDA receptor subunits, as has been found for the interaction of polyamines with the bacterial polyamine binding protein PotD (Kashiwagi et al., 1996b; Sugiyama et al., 1996). We previously found that mutations at NR1(E342), located in the center of the large amino terminal domain, and at NR1(D669) located in the M3-M4 loop influence spermine stimulation and proton inhibition (Williams et al., 1995; Kashiwagi et al., 1996a). In those studies we determined the effects of mutations at a total of 24 acidic residues in the amino terminal and M3-M4 loop regions of NR1. There are an additional 57 acidic residues in these domains (regions 1, 2, and 3 of Fig. 2A). We first examined D-to-N or E-to-Q mutations at each position (Figs. 1 and 2). Spermine stimulation was measured at NR1/NR2B receptors voltage-clamped at −20 mV to minimize voltage-dependent block by spermine (Figs. 1 and 2B). Voltage-dependent block was measured at NR1/NR2A receptors (which do not show spermine stimulation) in oocytes voltage-clamped at −100 mV (Fig. 2C).

Spermine stimulation was reduced by mutations at a cluster of acidic residues (D170, E181, E185, E186, E188) near the exon-5 splice site and at E297, D303, and D789 and was increased by mutations at D511 and D765 in NR1 (Fig. 2B). With the exception of D198N and E781Q, the NR1 mutants

had little or no effect on block by spermine (Fig. 2C). We also studied block by the high-affinity polyamine channel blocker N^1-dansyl-spermine (Chao et al., 1997). The profile seen with N^1-dansyl-spermine (1 μM, in oocytes voltage clamped at −70 mV) mirrored that seen for spermine block. Thus, mutations NR1(D198N) and NR1(E781Q) reduced block by N^1-dansyl-spermine, but other NR1 mutants had no effect (data not shown). We studied four other mutations at these positions—D198A, D198E, E781A, and E781D. These mutants had no effect on block by spermine or N^1-dansyl-spermine (data not shown). None of the mutants at D198 or E781 affected block by extracellular Mg^{2+} (data not shown). Because the D198N and E781Q mutations had only modest effects on polyamine block, these residues were not studied further. Because of the interactions between spermine, pH, and ifenprodil, we studied the influence of the NR1 mutants on ifenprodil inhibition (Figs. 1B and 2D). Mutations at the cluster of acidic residues (D170–E186) near the exon-5 splice site had only small effects on ifenprodil inhibition. However, mutation D130N abolished inhibition by ifenprodil (Figs. 1 and 2D).

Residues near Exon-5 Splice Site Influence Spermine Stimulation and Proton Inhibition. Spermine stimulation may involve relief of tonic proton inhibition (Traynelis et al., 1995). Therefore, we studied the pH sensitivity of mutants such as NR1(E181Q) that reduce spermine stimulation and the pH sensitivity of mutants at some nearby or adjacent residues (Figs. 3 and 4). Proton inhibition was reduced by mutations D170N, E181Q, E185Q, E297Q, D303N, and D789N in NR1 (Fig. 4B). In light of this, we studied other mutations at these positions to determine whether the presence of an acidic residue influences sensitivity to spermine and pH. We also studied the effects of multiple mutations in NR1 (e.g., E181Q plus E185Q) to determine if their effects were additive and mutations at the equivalent or nearby residues in NR2B.

At NR1(E181) and NR1(E185), mutations that neutralized the negative charge (E-to-Q) and/or reduced the size of the amino acid side chain (E-to-A) decreased spermine stimulation and proton inhibition. In contrast, E-to-D mutations at E181 or E185, which retain a negative charge, had no effect on sensitivity to spermine and pH (Fig. 3B and Fig. 4B).

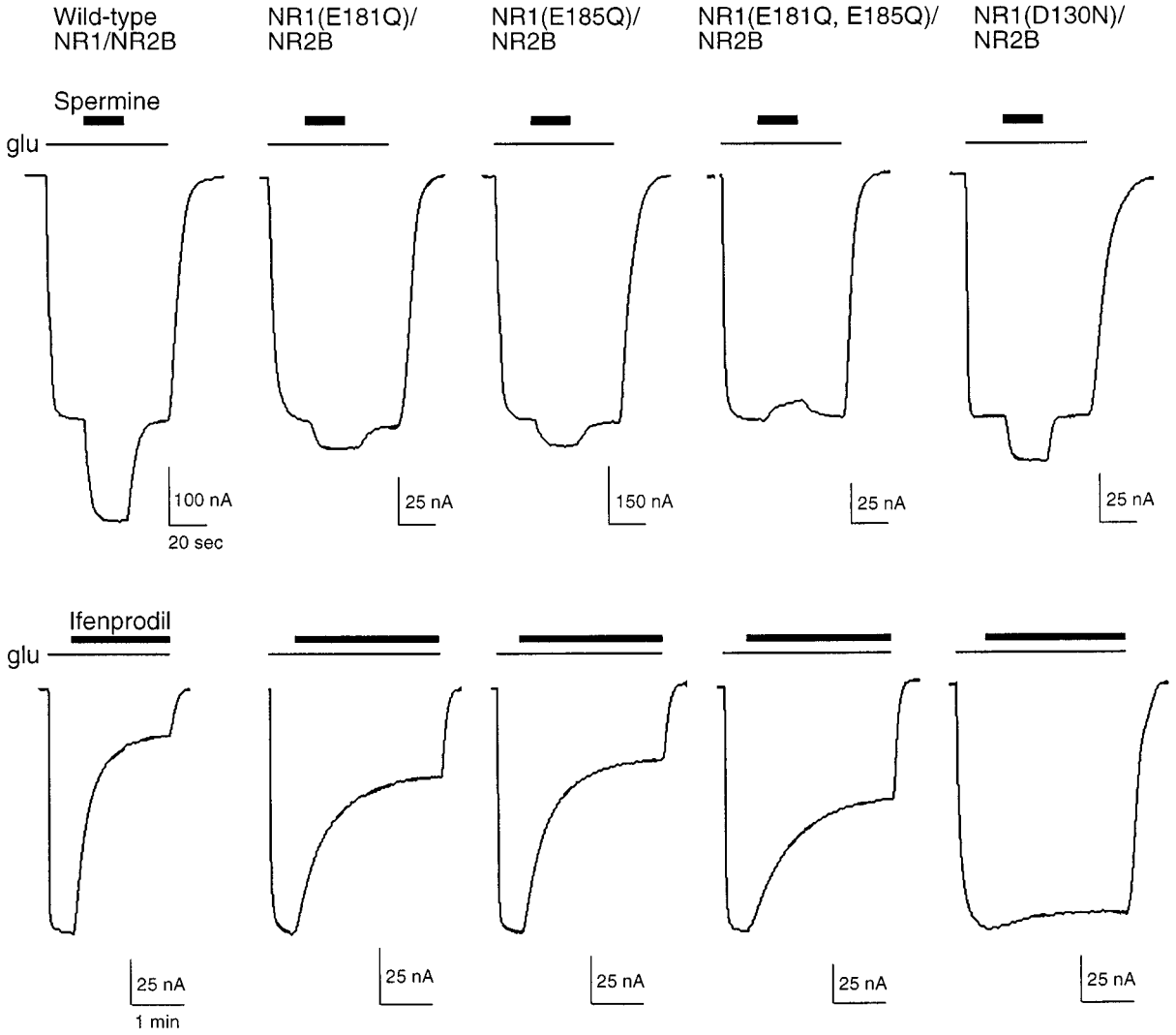
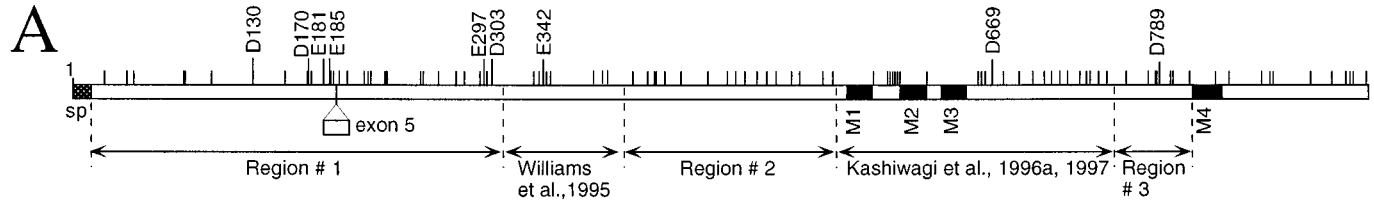
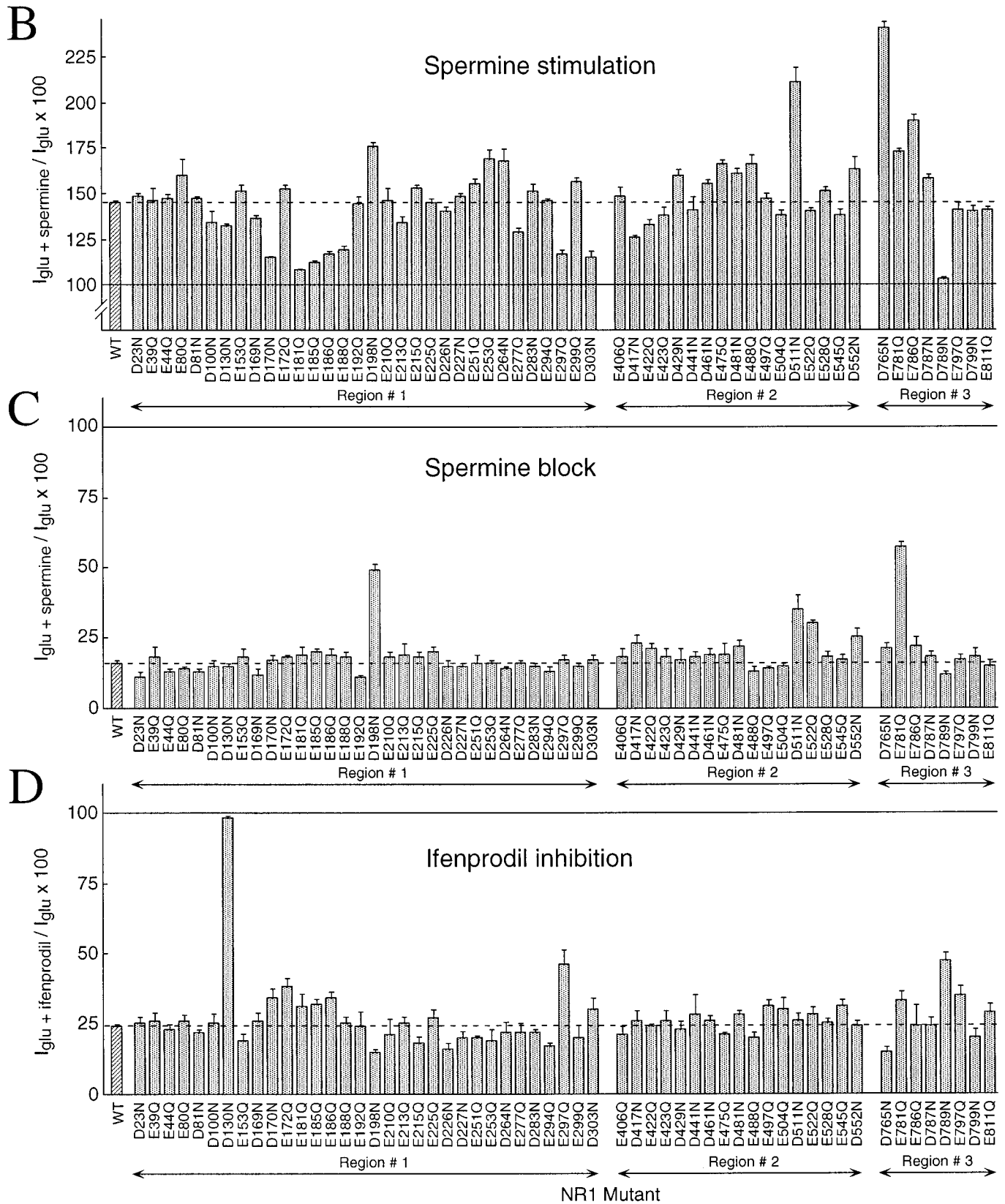


Fig. 1. Effects of NR1 mutants. Representative traces showing the protocols used to screen the effects of spermine (100 μM) and ifenprodil (1 μM) at wild-type and mutant receptors. NR1/NR2B receptors were studied in oocytes voltage-clamped at −20 mV and activated by glutamate (*glu*, 10 μM; with 10 μM glycine).





These data suggest that the presence of a negative charge at E181 and E185 is important for modulation by spermine and protons. A different profile was seen with mutations at D170, E297, D303, and D789. At these residues, E-to-A mutations had no effect (Fig. 4B), suggesting that the presence of an acidic moiety is not necessary for modulation by spermine and pH. Rather than directly altering the interaction with spermine or protons, mutations at these positions may interfere with the coupling of spermine and proton modulation or may have subtle, nonspecific effects on the structure or gating of the channel. In this regard, we found that a double mutation, NR1(E181Q, E297Q), did not have a larger effect than either of the single mutations (Fig. 4B). Mutations at E186 also had a profile different from those at E181 and E185. It may be that some mutations at E186 change the relative position of E181 and E185 and interfere with their interaction with spermine and protons. We also examined the pH sensitivity of receptors containing NR1(D511N) and NR1(D765N), which showed an increase in spermine stimulation (Fig. 2C). These mutations produced a small increase in pH sensitivity (pH IC_{50} = 7.5–7.6 compared with 7.3 at wild-type), which may explain the increase in spermine stimulation seen with these mutants.

A double mutant, NR1(E181Q, E185Q), had a larger effect than did either of the single mutants (Figs. 3C and 4B). Thus, the effects of mutations at E181 and E185 are additive, suggesting that they may contribute individually to a spermine-binding site. A triple mutant, NR1(E181Q, E185Q, E186Q), did not have a larger effect than the NR1(E181Q, E185Q) double mutant (Fig. 4B), consistent with the idea that E186 does not directly affect sensitivity to spermine and protons.

In NR2B there is an acidic residue, NR2B(E191), in a position analogous to NR1(E185) and there are several other acidic residues just downstream at positions NR2B(E198), NR2B(E200), and NR2B(E201) (Fig. 4A). Mutations at NR2B(E201) have previously been reported to affect sensitivity to spermine and pH (Gallagher et al., 1997). In NR2B, E-to-Q mutations at residues E191, E198, and E201 reduced spermine stimulation and proton inhibition (Fig. 4B). The largest effect was seen with NR2B(E191Q). The effects of mutations at NR2B(E191Q) were additive with those of mutations at E181 and E185 in NR1 (Fig. 4B).

We have previously found that positions E342 and D669 in NR1 are important determinants of sensitivity to spermine and pH (Williams et al., 1995; Kashiwagi et al., 1996a). To determine whether the effects of mutations at E342 and D669 in NR1 are additive with those of mutations at E181 and E185, we measured the pH sensitivity of receptors containing combinations of these mutants (Fig. 4C). The effects of mutations at NR1(E181) and NR1(E185) appear to be additive with those at NR1(E342) but not with those of NR1(D669).

Because the magnitude of spermine stimulation is dependent on agonist concentration (McGurk et al., 1990; Benveniste and Mayer, 1993; Williams, 1994, 1997), we studied glutamate and glycine sensitivity at the key mutants including D170N, E181Q, E185Q, the double E181Q/E185Q mutant, and E297Q in NR1, and E191Q and E198Q in NR2B. These mutants had no effect on sensitivity to glutamate and glycine. EC_{50} values ranged from 0.7 to 2.6 μM for glutamate and from 0.09 to 0.15 μM for glycine, compared with 1.3 μM (glutamate) and 0.13 μM (glycine) at wild-type receptors. Thus, changes in spermine sensitivity are not due to changes in agonist sensitivity.

Residues in the Proximal Amino-Terminal Domain Form An Ifenprodil-Binding Site. Mutations that reduced proton inhibition also produced small decreases in block by ifenprodil (data not shown, but see Fig. 1B for an example of the E181Q/E185Q double mutant), presumably due to an interaction between proton inhibition and ifenprodil inhibition. Indeed, such effects on ifenprodil sensitivity have been observed with mutations at NR1(E342) and NR1(D669), which also reduce pH sensitivity (Williams et al., 1995; Kashiwagi et al., 1996a; Mott et al., 1998). However, the NR1(D130N) mutation abolished ifenprodil inhibition without changing sensitivity to spermine and pH (Fig. 2D). It is conceivable that D130 is part of the ifenprodil-binding site. We therefore studied the effects of mutations at D130 in detail and made other mutations in a region encompassing 20 to 30 amino acids on each side of D130. Initially we screened mutations at 18 different residues in NR1 by measuring their effects on inhibition by 1 μM ifenprodil (Fig. 5A and B). In addition to D130, other residues that affected inhibition by ifenprodil were S108, Y109, F113, Y114, Y128, and H134. Most mutations in this region had no effect on spermine stimulation, although an increase in spermine stimulation together with a corresponding increase in pH sensitivity was seen with some mutants (Fig. 5, C and D). We also studied mutations at NR2B(D136), the position in NR2B that is equivalent to D130 in NR1, and at NR2B(H127). These mutations had no effect on sensitivity to ifenprodil (Fig. 5).

We subsequently studied several different mutations at the key residues near NR1(D130). Ifenprodil, acting at the high-affinity site on NR1/NR2B receptors, produces an incomplete block of macroscopic currents (Williams, 1993).

Fig. 1. Screening NR1 mutants. A, schematic of the NR1 subunit showing the positions of all the acidic (D and E) residues (thin vertical lines), the signal peptide (sp), the exon-5 insert site, and the membrane spanning or membrane re-entrant domains (M1-M4). The positions of the key acidic residues identified in this study (D130, D170, E181, E185, E297, D303, and D789) are shown together with residues (E342 and D669) previously found to influence sensitivity to spermine and protons. In this study, mutations were made at the D and E residues in regions 1, 2, and 3 of NR1. Mutations in the region around E342, in the M1 through M3 regions, and in the proximal two-thirds of the M3-M4 loop, which includes D669, have been reported previously (Williams et al., 1995; Kashiwagi et al., 1996a, 1997). B, effects of 100 μM spermine were determined in oocytes expressing NR1/NR2B receptors with wild-type (WT) or mutant NR1 subunits, voltage-clamped at −20 mV and activated by 10 μM glutamate and glycine. For the D481 mutant we used 100 μM (rather than 10 μM) glycine because this mutant reduces the potency of glycine by about 6-fold (Wafford et al., 1995). C, block by 10 μM spermine was measured in oocytes expressing NR1/NR2A receptors voltage-clamped at −100 mV. In (B) and (C), currents measured in the presence of spermine ($I_{glu\ +\ spermine}$) are expressed as a percentage of the control current (I_{glu}). D, effects of 1 μM ifenprodil were measured in oocytes expressing NR1/NR2B receptors voltage-clamped at −20 mV. Currents measured in the presence of ifenprodil are expressed as a percentage of the control current. The solid horizontal lines in (B), (C), and (D) represent the control current (I_{glu}, 100%) and the broken horizontal lines represent the mean effect of spermine or ifenprodil at wild-type receptors. Values are mean ± S.E.M. from 4 to 14 oocytes for each mutant and from 80 to 131 oocytes for wild-type receptors, which were studied in all batches of oocytes.

Therefore, concentration-inhibition curves were analyzed using the following equation:

$$I_{glu + ifen}/I_{glu} \times 100 =$$

$$100 - [In_{max}/(1 + \{IC_{50}/[ifenprodil]\}^{n}H)]$$

in which I_{glu} is the control response to glutamate, $I_{glu + ifen}$ is the response to glutamate measured in the presence of ifenprodil, In_{max} is the maximal percent inhibition caused by ifenprodil, IC_{50} is the concentration of ifenprodil producing 50% of that maximal inhibition, and nH is the Hill slope. A potential problem with this analysis is that ifenprodil can act as a voltage-dependent channel blocker at high concentrations, in addition to its effects as a voltage-independent, NR2B-selective antagonist (Williams, 1993). However, the voltage-dependent block is seen only at concentrations of 30 to 100 μM ifenprodil or greater and we therefore used ifenprodil only at concentrations up to 100 μM with oocytes voltage-clamped at −20 mV to minimize the voltage-dependent block. For some mutants that produced a large shift in ifenprodil sensitivity, it was not possible to determine the IC_{50} and maximum inhibition because there was no clear plateau in the concentration-inhibition curve.

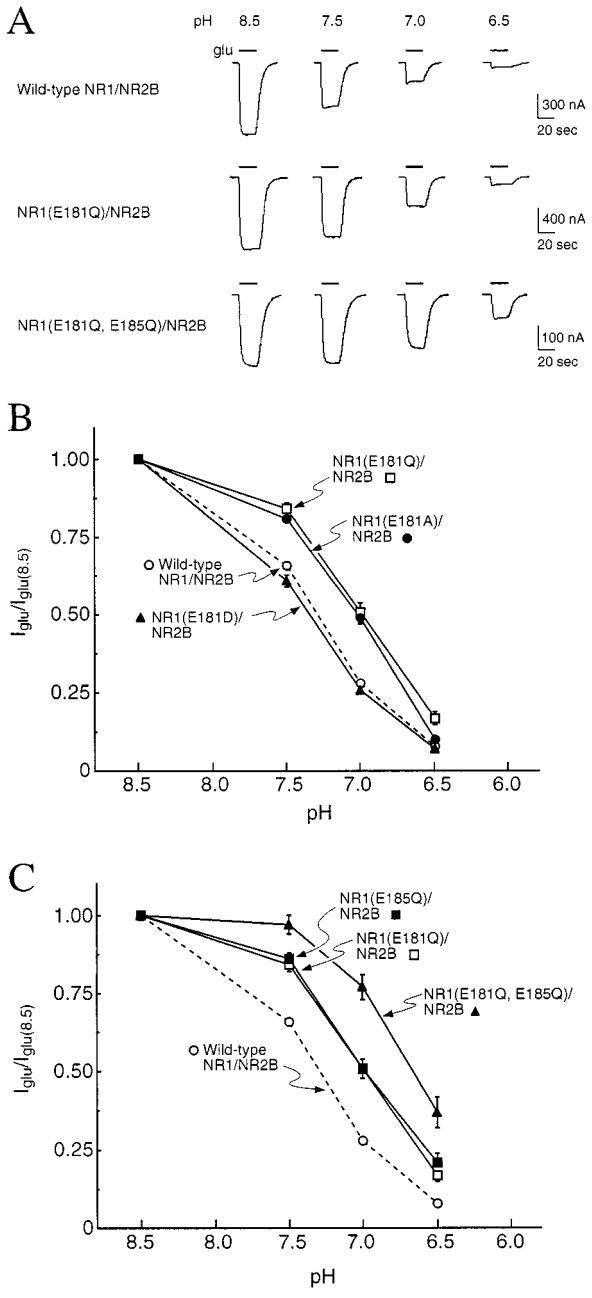


Fig. 3. Proton inhibition at mutant NMDA receptors. A, inward currents induced by glutamate (*glu*, 10 μM; with 10 glycine) at various extracellular pH (8.5–6.5) in oocytes expressing wild-type and mutant NMDA receptors. B and C, proton inhibition curves were constructed by measuring responses to glutamate and glycine at different extracellular pH using protocols similar to those shown in (A). Values are mean ± S.E.M. from 5 to 20 oocytes for each mutant and from 89 oocytes for wild-type.

Mutations at S108 through H134 in NR1 had complex effects on inhibition by ifenprodil (Figs. 6 and 7, Table 1). Some mutations changed the potency and/or maximum effect of ifenprodil whereas other mutations abolished inhibition or even showed an ifenprodil-induced potentiation of NMDA currents. Examples of mutants that reduced the potency but did not change the maximum effect are those at NR1(Y114) (Fig. 6C, Table 1). Mutations that reduced the potency of ifenprodil also increased the rate of onset and recovery of inhibition. Examples are shown for Y114A and Y114L in Fig. 6A. We studied three mutations at F113, adjacent to Y114, and found that F113L drastically reduced ifenprodil block whereas F113A and F113Y had no effect. Thus, the presence of an aromatic group (F or Y) at positions F113 and Y114 does not in itself seem to be critical for ifenprodil inhibition because F-to-A or Y-to-A mutations have only modest effects (Table 1). It may be that the F-to-L and Y-to-L mutations at these positions alter the hydrophobicity of this region of the ifenprodil binding site, or that the leucine side chain directly interferes with ifenprodil binding, perhaps by steric hindrance. Upstream from these positions, residues S108 and Y109 have a pronounced effect on ifenprodil sensitivity. Replacement with a hydrophobic leucine residue (S108L) rather than the polar serine at S108 drastically reduced ifenprodil sensitivity. The presence of alanine (S108A) or threonine (S108T) produced a smaller shift in the potency of ifenprodil and S108A reduced the maximum inhibition by about 30% (Table 1). Thus, at S108, the presence of a hydroxyl group (found in S and T) may be important for the maximum effect and the relative size of the side chain may be important for affinity.

We studied six different mutants at Y109 to try to define more clearly the role of this residue in ifenprodil binding. At receptors containing Y109W (data not shown) and Y109L (Fig. 6B), ifenprodil potentiated rather than inhibited NMDA currents. Mutation Y109V produced a large decrease in potency, whereas Y109A and Y109F produced 20- to 30-fold changes in potency and Y109S had only a small effect (Table 1). In addition to its effect on the potency of ifenprodil, the Y109F mutation, in which the hydroxyl group is removed from the aromatic moiety, reduced the maximum effect to about 40% of that seen at wild-type receptors (Table 1; Fig. 6B).

At NR1(D130), the D-to-N and D-to-A mutations reduced the potency of ifenprodil, whereas a D-to-E mutation reduced the maximum effect (Fig. 7; Table 1). It is possible that the presence of an acidic group (D or E) at this position is important for affinity (compare the wild-type D residue with mutations N, A, and E), whereas the size of the side chain

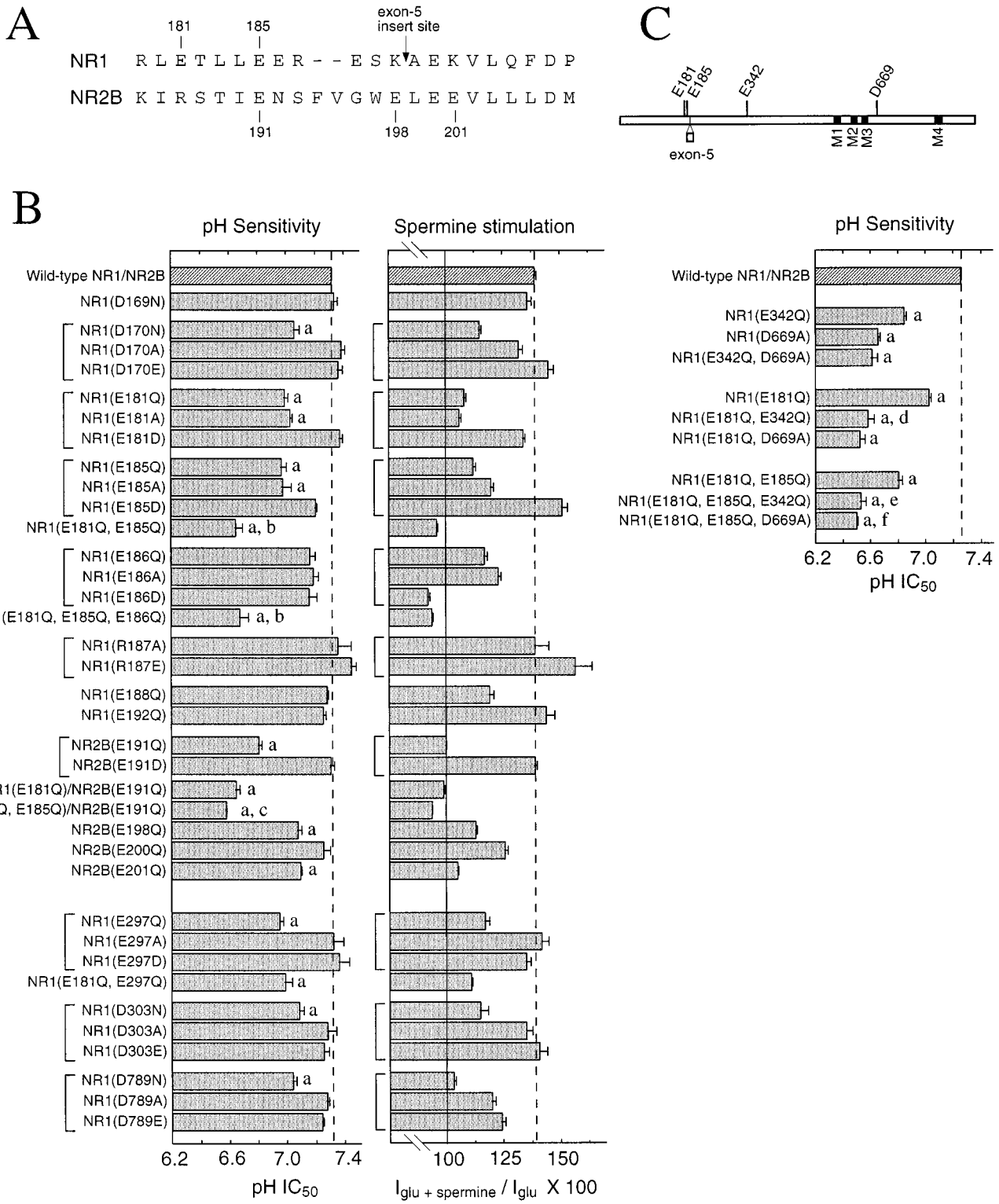


Fig. 4. Spermine stimulation and proton inhibition at mutant NMDA receptors. A, sequences of NR1 and NR2B that flank residues NR1(E181) and NR1(E185). Amino acids are numbered from the initiator methionines in NR1 and NR2B, and sequences are aligned as in Ishii et al. (1993). B and C, spermine stimulation was measured using 100 μM spermine, with 10 μM glutamate and glycine, in oocytes expressing NR1/NR2B receptors and voltage-clamped at −20 mV. The pH IC_{50} was determined by measuring responses to glutamate (10 μM; with 10 μM glycine) at different extracellular pH (see Fig. 3). Unless otherwise shown, mutant NR1 subunits were expressed together with wild-type NR2B, and mutant NR2B subunits were expressed together with wild-type NR1. Values are mean ± S.E.M. from 4 to 20 oocytes for each mutant and from 17 to 111 oocytes for wild-type. a, $P < .05$ compared with wild-type; b, $p < .01$ compared with NR1(E181Q) or NR1(E185Q); c, $p < .05$ compared with NR1(E181Q, E185Q) or NR2B(E191Q); d, $p < .01$ compared with NR1(E342Q) or NR1(E181Q); e, $p < .01$ compared with NR1(E342Q) or NR1(E181Q, E185Q); f, $p < .05$ compared with NR1(D669A) or NR1(E181Q, E185Q); one way ANOVA with post hoc Tukey-Kramer multiple comparisons test. The schematic in (C) shows the relative positions of residues E181, E185, E342, and D669 in NR1.

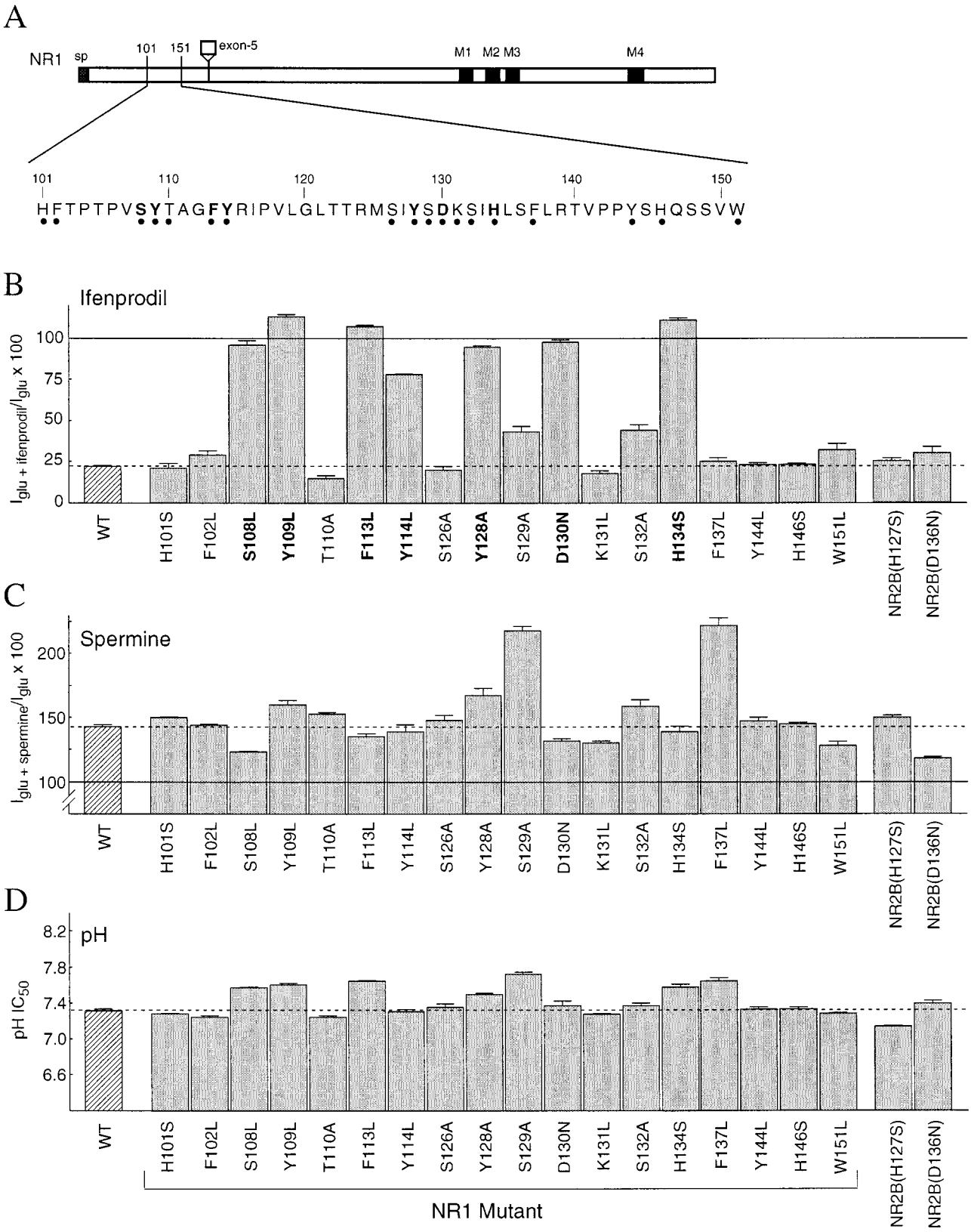


Fig. 5. Screening NR1 mutants near NR1(D130). A, schematic of the NR1 subunit showing the relative positions of the exon-5 splice site, the M1–M4 regions, and the region between amino acids 101 and 151 in which mutants were made. *sp*, signal peptide. The amino acid sequence in this region is shown below the schematic. The positions at which mutations were made are indicated by a circle, and residues at which mutations have a pronounced effect on ifenprodil sensitivity are shown in bold. B, effects of 1 μM ifenprodil were measured in oocytes expressing NR1/NR2B receptors with wild-type (WT) or mutant NR1 and NR2B subunits, activated by 10 μM glutamate and glycine, and voltage-clamped at −20 mV. Currents measured in the presence of ifenprodil ($I_{glu\ +\ ifenprodil}$) are expressed as a percentage of the control current (I_{glu}). C, effects of 100 μM spermine were determined in oocytes voltage-clamped at −20 mV and activated by 10 μM glutamate and glycine. D, pH IC_{50} was determined by measuring responses to glutamate (10 μM; with 10 μM glycine) at different extracellular pH using protocols similar to those shown in Fig. 3. Values are mean ± S.E.M. from 4 to 12 oocytes for each mutant and from 36 to 61 oocytes for wild-type receptors, which were studied in all batches of oocytes.

(compare D with E) controls the maximum inhibition (Fig. 7; Table 1). Mutations at NR1(Y128) also had a large effect on ifenprodil sensitivity.

Although ifenprodil is not a competitive antagonist at the glutamate or glycine sites, ifenprodil inhibition is dependent on the concentrations of glutamate and glycine used to activate NMDA receptors (Legendre and Westbrook, 1991; Williams, 1993; Kew et al., 1996). Ifenprodil inhibition is also dependent on extracellular pH (Pahk and Williams, 1997; Mott et al., 1998). We therefore determined the EC_{50} for glutamate and glycine and the pH IC_{50} at all of the mutants listed in Table 1. The results are shown in Fig. 8, together with the IC_{50} and In_{max} values for ifenprodil. None of the mutants decreased agonist potency and, with the exception of NR1(Y109A), there were only small effects on pH sensitivity (Fig. 8, C and D). Thus, a decrease in sensitivity to pH or to glutamate cannot account for the effects of these mutants on sensitivity to ifenprodil. To further investigate the specificity of the mutants, we studied two other hallmark features of NMDA receptors—permeability of Ba^{2+} and voltage-dependent block by extracellular Mg^{2+}. Ba^{2+} permeability was assessed by measuring the shift in the reversal potential in Ba^{2+}-saline compared with that in Na^{+}-saline. At wild-type NR1/NR2B receptors the shift in reversal potential was $+20 \pm 1$ mV (18 oocytes). Voltage-dependent Mg^{2+} block was determined by using 100 μM Mg^{2+} in oocytes voltage-clamped at -20 and at -70 mV. None of the mutants altered Ba^{2+} permeability or Mg^{2+} block (Fig. 8, E and F).

The NR1(Y109A) mutant drastically reduced pH sensitivity (Fig. 8C). We therefore determined whether the effects of this mutant on ifenprodil inhibition could be due to a change in pH sensitivity, because ifenprodil inhibition is pH-sensitive. We measured concentration-inhibition curves for ifenprodil at pH 7.5 (near the pH IC_{50} at wild-type) and at pH 6.5 (near the pH IC_{50} at Y109A) at receptors containing the Y109A mutant. Surprisingly, there was no difference in the potency or efficacy of ifenprodil at pH 6.5 versus pH 7.5 with this mutant (Table 1). At wild-type receptors, there is about a 15-fold shift in the potency of ifenprodil over an equivalent range of pH sensitivity (Pahk and Williams, 1997; Mott et al., 1998). This suggests that the Y109A mutant disrupts the coupling of proton inhibition and ifenprodil inhibition.

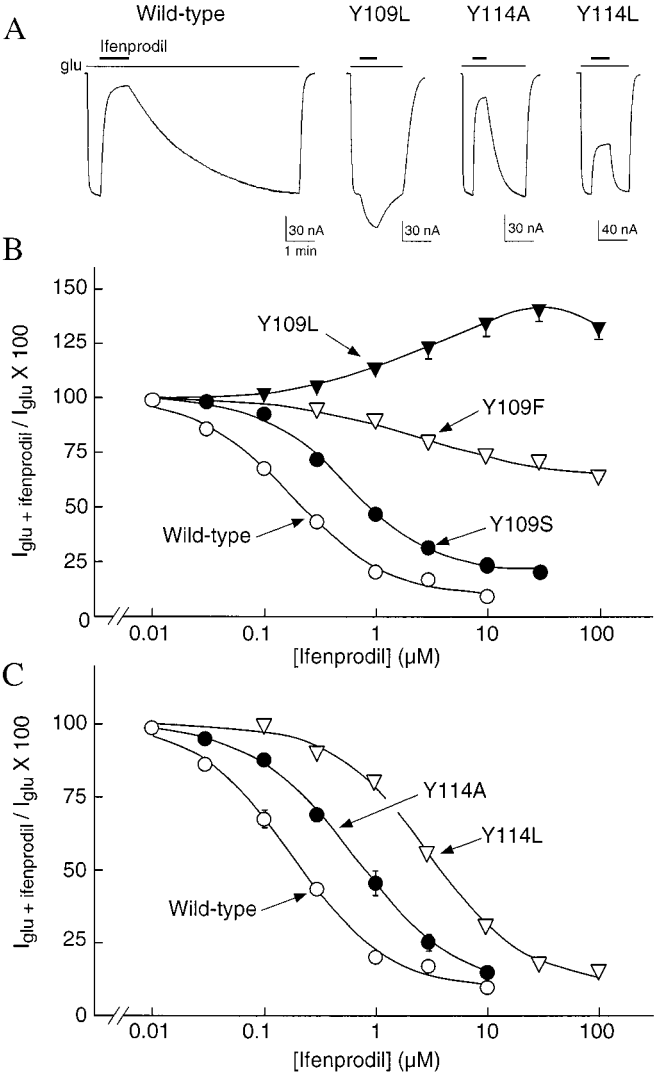


Fig. 6. Effects of ifenprodil at mutant NMDA receptors. A, representative traces showing the effects of 3 μM ifenprodil at wild-type and mutant NR1/NR2B receptors activated by 10 μM glutamate and glycine (*glu*) and voltage-clamped at -20 mV. B, concentration-effect curves for ifenprodil were determined at NR1/NR2B receptors containing wild-type and mutant NR1 subunits using protocols similar to those shown in (A). Values are mean ± S.E.M. from 3 to 18 oocytes at each concentration of ifenprodil.

Discussion

Sensitivity to Spermine, pH, and Ifenprodil. We have previously identified several acidic residues in the extracellular domains of NR1, including E342 and residues in the pore-forming region of this subunit, that influence sensitivity to spermine and protons (Williams et al., 1995; Kashiwagi et al., 1996a, 1997). In this paper we have identified additional residues that control sensitivity to spermine and pH. A common feature of mutations at these residues is that they produce a decrease in spermine stimulation with a concomitant decrease in pH sensitivity. At residues E181, E185, and E342 in NR1, a negative charge is important for modulation by spermine and pH. Another salient feature of mutations at these positions is that their effects are additive. We suggest that the acidic residues where a negative charge is important

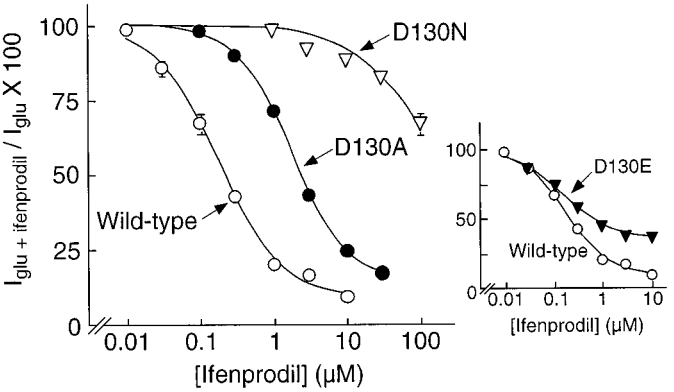


Fig. 7. Effects of NR1(D130) mutants. Concentration-effect curves for ifenprodil were determined at NR1/NR2B receptors containing wild-type and mutant NR1 subunits in oocytes voltage-clamped at -20 mV and activated by 10 μM glutamate and glycine. Values are mean ± S.E.M. from 4 to 10 oocytes at each concentration of ifenprodil.

may interact directly with one or more of the amino groups of spermine.

Ifenprodil is an NMDA receptor antagonist that selectively inhibits receptors containing NR2B (Williams, 1993; Williams et al., 1993). Ifenprodil is also a potent antagonist at homomeric NR1 receptors expressed in oocytes (Williams et al., 1993) suggesting that the high-affinity ifenprodil site is located on the NR1 subunit, although it is possible that "homomeric" NR1 receptors expressed in oocytes include an NR2-like subunit that is endogenous to oocytes (Soloviev and Barnard, 1997). We have identified seven residues in NR1 that appear to form part of the ifenprodil binding site. A complication of these experiments was that some mutations produce a stimulation, rather than inhibition, by ifenprodil. The only conditions under which ifenprodil has been previously reported to stimulate NMDA responses were with low concentrations of glutamate, because ifenprodil increases agonist affinity (Kew et al., 1996). However, all of our experiments were carried out with saturating concentrations of glutamate, suggesting that a mechanism other than an increase in agonist affinity is responsible for the ifenprodil stimulation. Because ifenprodil may act to allosterically reduce channel gating (Legendre and Westbrook, 1991; Kew et al., 1996) and/or to increase proton inhibition (Mott et al., 1998), it may be that some mutations alter the coupling of the ifenprodil site in such a way that ifenprodil behaves in the opposite manner, i.e., it increases channel gating or reduces proton inhibition with those mutants. Another possibility is that ifenprodil stimulation is mediated through a separate, low-affinity binding site and the stimulation is unmasked at mutants such as Y109L in which there is an enormous decrease in the potency of ifenprodil inhibition.

TABLE 1

Effects of ifenprodil at mutant NMDA receptors

The results are from experiments with NR1/NR2B receptors containing wild-type or mutant NR1 subunits in oocytes voltage-clamped at −20 mV. Values for the IC_{50}, Hill-slope (nH), and maximal inhibition (In_{max}) were derived from concentration-inhibition curves with ifenprodil (see Figs. 6 and 7).

NR1 Mutant	IC_{50}	Mutant/wild-type[a]	nH	In_{max}	Mutant/wild-type[a]
	μM			%	
Wild-type	0.17		1.0	90	
S108L[b]	>100	>500			
S108A	1.9	11	0.9	65	0.7
S108T	2.6	15	1.1	88	1.0
Y109F	3.0	18	0.7	39	0.4
Y109S	0.54	3	1.1	80	0.9
Y109A (pH 7.5)[c]	5.5	32	1.2	93	1.0
Y109A (pH 6.5)[c]	5.1	30	1.2	96	1.1
Y109L[d]	>100	>500			
Y109V[b]	>100	>500			
Y109W[d]	>100	>500			
F113L[b]	>100	>500			
F113A	0.21	1	1.1	91	1.0
F113Y	0.06	0.4	1.0	85	0.9
Y114L	2.9	17	1.0	89	1.0
Y114A	0.64	4	0.9	92	1.0
Y114F	0.71	4	0.9	94	1.0
Y128A	22	129	0.8	47	0.5
Y128L[e]	NF				
D130N[b]	>100	>500			
D130A	1.7	10	1.2	85	0.9
D130E	0.16	1	0.9	65	0.7
H134S[d]	>100	>500			

[a] Ratio of the IC_{50} or maximal inhibition (In_{max}) at mutant/wild-type receptors.
[b] 100 μM Ifenprodil inhibited NR1(S108L)/NR2B by 43 ± 1%, NR1(Y109V)/NR2B by 45 ± 2%, NR1(F113L)/NR2B by 10 ± 1%, and NR1(D130N)/NR2B by 33 ± 4%.
[c] Mutation Y109A produced a large decrease in pH sensitivity, reducing the pH IC_{50} from 7.3 (wild-type) to 6.3 (Y109A) (see Fig. 8). The other mutants had little or no effect on pH sensitivity. Because ifenprodil inhibition is pH sensitive (Pahk and Williams, 1997), we studied the effects of Y109A at pH 7.5 (close to the pH IC_{50} of wild-type receptors) and at pH 6.5 (close to the pH IC_{50} of Y109A). All other data in this table are from experiments carried out at pH 7.5.
[d] Ifenprodil (1–100 μM) produced a stimulation at NR1(Y109L)/NR2B receptors (31 ± 4% at 100 μM) and at NR1(H134S)/NR2B receptors (42 ± 3% at 100 μM), and a concentration-dependent stimulation (37 ± 6% at 10 μM) or inhibition (11 ± 1% at 100 μM) at NR1(Y109W)/NR2B receptors.
[e] NF, non-functional. NR1(Y128L)/NR2B receptors (two independent Y128L mutants) gave no response or very small responses (1–2 nA) to glutamate and glycine (10 μM; pH 7.5). This was not due to an increase in tonic proton inhibition, because there was no response at pH 9.0, nor to a decrease in sensitivity to agonists, because there was no response to 1 mM glutamate and glycine.

A Model of the R1–R2 Domain in NR1. The S1 and S2 regions of glutamate receptor subunits have homology with bacterial periplasmic amino acid binding proteins including lysine/arginine/ornithine binding protein (LAOBP) and glutamine binding protein (QBP; Sutcliffe et al., 1996). It was proposed that the S1 and S2 regions have a tertiary structure similar to QBP and LAOBP and form the agonist binding pocket (Kuryatov et al., 1994; Sutcliffe et al., 1996). Recently, the crystal structure of an S1–S2 fusion protein of the GluR2 subunit was solved at 1.9 Å resolution (Armstrong et al., 1998). The S1–S2 fusion protein indeed has a structure similar to that of QBP, with the agonist binding site being located between the S1 and S2 lobes.

Models for the region that precedes S1 are not available. However, it has been noted that this region shows homology with several other bacterial periplasmic binding proteins, in particular LIVBP (Sutcliffe et al., 1996) and the polyamine binding protein PotD (Williams et al., 1995). We compared the predicted secondary structure of the region containing amino acids 19 (the start of the mature peptide) to 400 of NR1 with 1,400 different proteins of known structure using the LIBRA I program (Ota and Nishikawa, 1997). The greatest similarity was found between NR1(22–375) and LIVBP (Fig. 9A). Although NR1(22–375) shares only 15% amino acid identity with LIVBP there is a remarkable degree of overlap in the putative secondary structure of this region of NR1 with that of LIVBP (Fig. 9A).

The structure of LIVBP, determined by X-ray crystallography, is a globular protein of two domains, with a central cleft that forms the amino acid binding site, similar to that of other bacterial periplasmic binding proteins (Sack et al., 1989). Therefore, based on the predicted structural homology of NR1(22–375) with LIVBP, we propose that the tertiary structure of this region of NR1 is similar to that of LIVBP (Fig. 9). We propose that this region of NR1 contains two domains and we refer to these as "regulatory (R) domains" R1 and R2. Thus, the R1–R2 segment precedes S1 and may have a "clamshell" structure similar to the S1–S2 domain (Fig. 9B). To aid in modeling the possible location of the binding sites for spermine and ifenprodil, we have mapped the critical residues uncovered in this study and in a previous study (Williams et al., 1995) onto a projected structure of LIVBP (Fig. 9B). Residues that form part of the ifenprodil binding site lie on the surface or within a small pocket in the center of the R1 domain. This pocket is predicted to have a size of about 6 Å × 10 Å and a depth of 3 to 4 Å. The pharmacophore that has been proposed for the ifenprodil binding site involves a separation of about 8 Å between the nitrogen and the phenolic hydroxyl and 10 Å between the nitrogen and the unsubstituted aromatic ring of ifenprodil (i.e., an overall length of about 18 Å; Tamiz et al., 1998). It is conceivable that the region involving residues 109 to 134 in R1 is in-

volved in binding the nitrogen and phenolic ring of ifenprodil. Residues S108, Y109, and D130 may interact with the nitrogen, whereas F113 and Y114 may form part of a hydrophobic pocket and/or interact with the aromatic ring of ifenprodil by π-stacking. A problem with modeling the residues in this region is that S108 and Y109 lie in a section where there is limited overlap in the secondary structure of NR1 and LIVBP, and Y128 and D130 lie on a linker between two α helices. Nonetheless, the close proximity of the seven critical residues is consistent with the idea that this region forms part of a binding pocket for ifenprodil. The other end of the ifenprodil molecule presumably interacts with a second hydrophobic pocket (Tamiz et al., 1998). We have not yet identified this pocket, but it could lie in the R2 domain of NR1 or it could lie in the NR2B subunit. If it is located in the R2 region of NR1, it is possible that this region overlaps with a site that forms a hydrophobic interaction with the backbone of spermine, accounting for the apparent interactions between spermine and ifenprodil (Carter et al., 1990).

The acidic residues that are critical for spermine stimulation and pH inhibition (E181, E185, D339, and E342) are located on helix *V* near the top of the R2 segment and on the loop connecting R1 and R2. Other residues that influence spermine and pH modulation (D170, E297, and D303) are located on the linker before helix *V* and in the region near helix *IX*, which may not be on the surface of the protein. It is conceivable that one amino group of spermine could interact with residues E339 and E342 and a second amino group could interact with residues E181 and E185. Because spermidine (a triamine) but not putrescine (a diamine) can potentiate NMDA receptors, a third amine recognition site is also necessary. This could involve D170, D669 or, perhaps, residues in the NR2B subunit. The exon-5 insert, which may function as a constitutive spermine-like modulator, would be

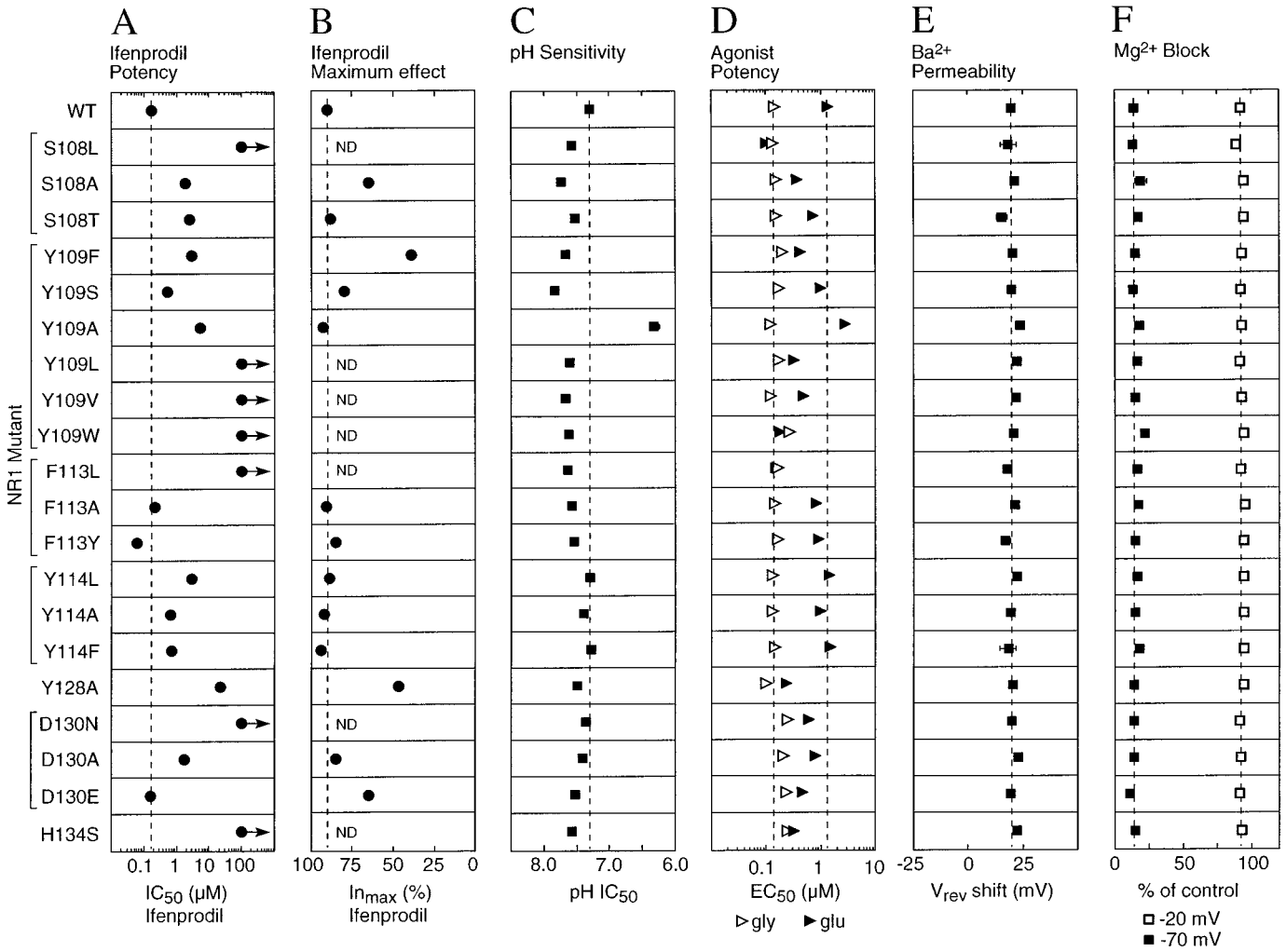


Fig. 8. Properties of receptors containing NR1 mutants. All results are from experiments using NR1/NR2B receptors containing wild-type (WT) or mutant NR1 subunits. A and B, data for the potency (IC_{50}) and maximal inhibition (In_{max}) of ifenprodil are from Table 1. For mutants where the IC_{50} is unknown, but is >100 μM, a symbol with an arrow is drawn at 100 μM; ND, not determined. C, pH IC_{50} was determined using protocols similar to those shown in Fig. 3. Values are mean ± S.E.M. from 3 to 12 oocytes for each mutant. D, EC_{50} values for glutamate (*glu*) and glycine (*gly*) were determined from concentration-response curves for each agonist. Glutamate curves were measured in the presence of 10 μM glycine, and glycine curves were measured in the presence of 10 μM glutamate. Values are the geometric mean from three to five oocytes for each mutant. E, shift in reversal potential (V_{rev}) was calculated as the difference in V_{rev} measured in Na^+ saline (96 mM NaCl, 1.8 mM $BaCl_2$) compared with that in Ba^{2+}-saline (64 mM $BaCl_2$). F, effects of extracellular Mg^{2+} (100 μM) on responses to glutamate (10 μM; with 10 μM glycine) were determined in oocytes voltage-clamped at −20 mV and at −70 mV. Currents measured in the presence of Mg^{2+} are expressed as a percentage of the control current at each holding potential. Values in (E) and (F) are mean ± S.E.M. from three to ten oocytes for each mutant. In all panels, the broken vertical lines indicate the values measured in wild-type receptors.

positioned in the R2 domain of NR1 between helix *V* and sheet G (Fig. 9B). The exon-5 insert contains 21 amino acids and would be slightly larger than helix *V*. This is consistent with the idea that the exon-5 insert acts to shield the spermine binding site or interacts directly with that site (Traynelis et al., 1995).

The putative structure of the R1–R2 domain has important implications for understanding the function of glutamate receptors. The amino acid binding proteins such as LAOBP and LIVBP can exist in an open (unliganded) and closed (liganded) form. This is also the case for the amino acid binding sites formed by S1–S2 in glutamate receptors (Armstrong et al., 1998). If the same is true of the R1–R2 domains, it begs two important questions—what (if anything) is the nature of the ligand that binds within the cleft of the R1–R2 domain and how does this domain affect the gating of glutamate receptor channels? Potential ligands for the R1–R2 cleft include spermine, Mg^{2+} (which can act at the stimulatory spermine site), Zn^{2+},

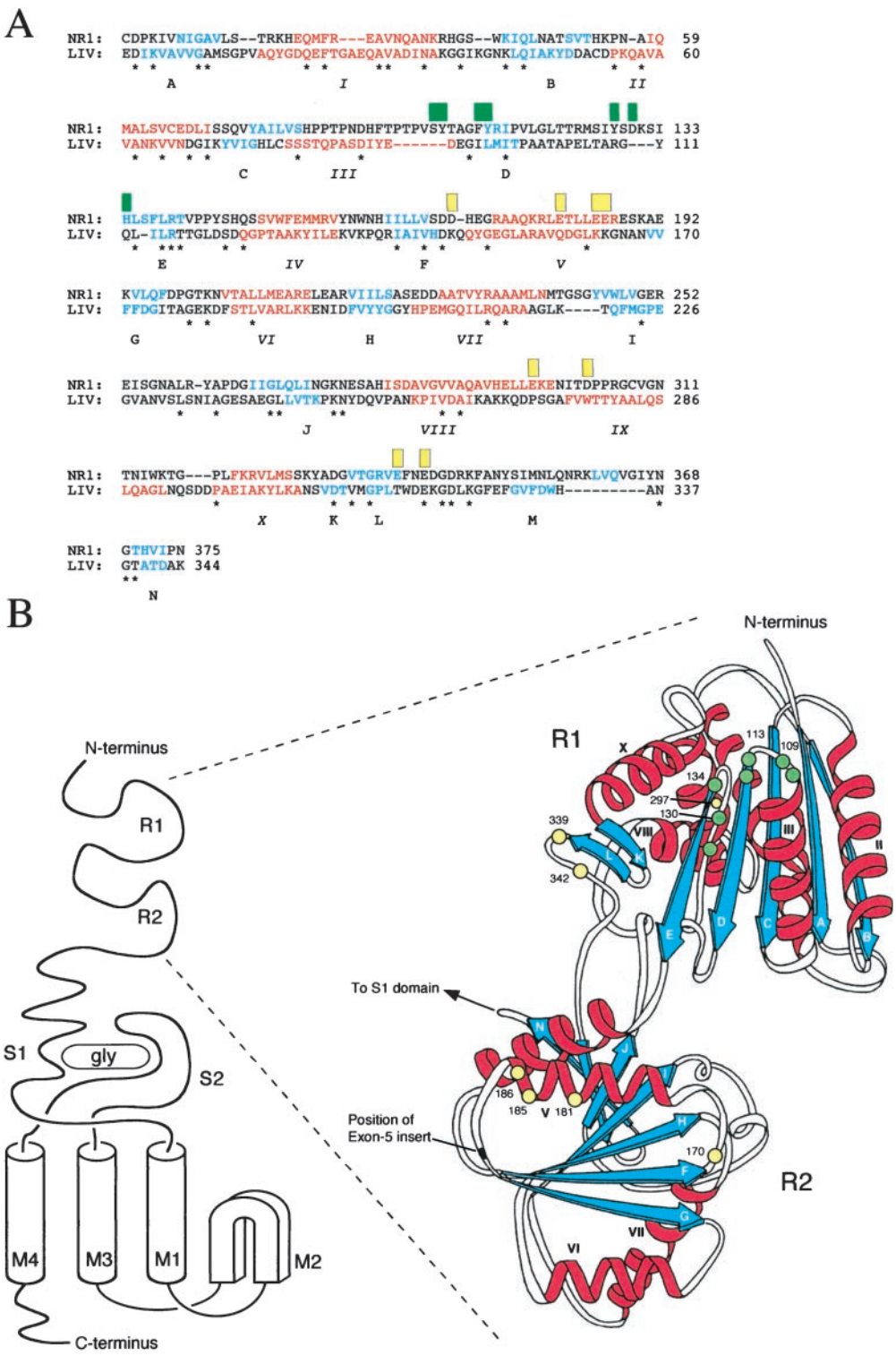


Fig. 9. Modeling the amino terminal domain of NR1. A, amino acid sequences of NR1(22–375) and LIVBP (LIV) are aligned for maximum homology. Identical residues are indicated by stars below the LIV sequence. The positions of α helices and β sheets are shown in red and blue, respectively. For LIVBP, the distribution of α helices and β sheets corresponds to that in the crystal structure of LIVBP (Sack et al., 1989). The α helices in LIVBP are labeled *I* through *X*, and the β sheets are labeled A through N. For NR1, the predicted positions of α helices and β sheets were determined by computer modeling using the LIBRA I program (Ota and Nishikawa, 1997). B, schematic shows the relative positions of the glycine binding domain (S1–S2), the pore-forming loop (M2) and membrane-spanning domains (M1, M3, and M4), and the R1–R2 segment preceding S1. The exploded view of R1–R2 shows a projected structure of LIVBP based on the known crystal structure of LIVBP (modified from Sack et al., 1989). In (A) and (B) the locations of residues that are involved in ifenprodil inhibition are shown in green and residues that influence sensitivity to spermine and pH are shown in yellow.

and other endogenous ligands. It is interesting to speculate that the R1–R2 domains in different glutamate receptor types may have binding pockets for different endogenous modulators. Because the R1–R2 region is located upstream of one-half of the agonist binding pocket, it is conceivable that movements or conformational changes within the R1–R2 region influence the coupling of S1–S2 to channel gating, consistent with the pronounced effects of spermine, pH, and ifenprodil on channel opening and the complex interactions between these three regulatory molecules.

Acknowledgments

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