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Pharmacological characterization of interactions of RO 25-6981 with the NR2B (ε2) subunit

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Abstract

We used ligand binding to ascertain whether the pharmacological actions of RO 25-6981 [(*R*:(*), *S*:(*))-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol] match those of other NR2B (ε2) subunit specific agents. RO 25-6981 inhibited binding of ^{125}I-MK801 [iodo-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohept-5,10-imine maleate] to receptors made from NR1a/ε2 but not NR1a/ε1. Increasing the concentration of spermidine did not change the efficacy of RO 25-6981 and minimally changed the IC_{50} value. Chimeric ε1/ε2 receptors demonstrated that the structural determinants for high affinity actions of RO 25-6981 were contained completely within the first 464 amino acids, but no receptor retained wildtype features when the size of the ε2 component was decreased further. ε1Q336R receptors were more inhibited by ifenprodil and RO 25-9681 than wildtype ε1 receptors in ligand binding assays but not in functional assays. Selected mutations of ε2E200 and ε2E201 also decreased the sensitivity of receptors to ifenprodil and RO 25-6981. These results suggest that RO 25-6981 shares structural determinants with ifenprodil and other modulators in the NR2B subunit.

Keywords: Glutamate; Stroke; Ifenprodil; Polyamine

1. Introduction

The NMDA receptor is a glutamate-gated ion channel that is crucial in the processes of long term potentiation and excitotoxicity (Collingridge and Lester, 1989; Choi and Rothman, 1990). Consequently, the NMDA receptor is the focus of drug development for therapy and prevention of numerous neurological and psychiatric disorders. This receptor is assembled from combinations of NR1 and NR2 subunits, with at least one of each being necessary for a functional receptor. Eight different NR1 splice variants have been characterized, and four different NR2 subunits are made as products of distinct genes (Meguro et al., 1992; Monyer et al., 1992; Moriyoshi et al., 1991; Ishii et al., 1993). Murine forms of these subunits have also been characterized, and demonstrate a high level of homology with rat subunits (Kutsuwada et al., 1992; Ikeda et al., 1992). Murine NR2 subunits (called ε1–ε4) coassemble with rat NR1 subunits to produce receptors with properties identical to those of receptors from purely rat subunits (Chazot et al., 1994; Gallagher et al., 1996).

Different combinations of NR1 and NR2 subunits produce receptors with distinct pharmacological and biochemical properties. Several agents are selective or specific for receptors, which contain the NR2B subunit. These drugs include polyamines such as spermidine and spermine, which potentiate NMDA receptor activity at low micromolar concentrations, and the noncompetitive antagonists ifenprodil and haloperidol (Williams, 1993; Williams et al., 1994; Lynch et al., 1995; Lynch and Gallagher, 1996; Coughenour and Cordon, 1997; Ilyin et al., 1996). Pharmacological experiments and approaches using site directed mutagenesis have demonstrated overlap between the struc-

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tural determinants for these agents as well as determinants or conditions that are modulator selective (Brimecombe et al., 1998; Gallagher et al., 1996, 1997, 1998; Pahk and Williams, 1997; Masuko et al., 1999). These agents also overlap structurally and mechanistically with the proton sensor of the NMDA receptor, although specific structural components and pH dependence may differentiate these sites (Traynelis et al., 1995; Gallagher et al., 1997; Pahk and Williams, 1997; Mott et al., 1998; Masuko et al., 1999).

Novel agents containing structural similarity to ifenprodil and haloperidol such as CP101,606 [(R^*,R^*)-4-hydroxy-(4-hydroxyphenyl)-β-methyl-4-phenyl-1-piperidine-ethanol] and RO 25-6981 [(R:(*),S:(*))-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol [(+/−)-Ro 25-6981] have recently been developed as NR2B subunit-specific antagonists (Brimecombe et al., 1998; Chenard et al., 1995; Mutel et al. 1998; Tamiz et al., 1998; Butler et al., 1998). Understanding their mechanisms of action is important for continued development of these agents as neuroprotective and antiparkinsonian agents. In the present study, we have confirmed the selectivity of one of these agents, RO 25-6981, for the NR2B subunit in a commonly used ligand-binding assay for NMDA receptors. In addition, we have used chimeric NR2 subunits and site directed mutants of NR2 subunits to investigate the NR2B subunit-selective structural determinants for action for RO 25-6981.

2. Materials and methods

2.1. Materials

Haloperidol, (+)-MK 801 (hydrogen maleate) [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohept-5,10-imine maleate], and ifenprodil (tartrate) were obtained from Research Biochemicals International. Spermidine, polyethylenimine, and HEPES were products of Sigma. Restriction enzymes and Taq DNA polymerase were purchased from either GIBCO BRL or New England Biolabs. Deoxynucleotide triphosphates used in PCR applications were bought from Pharmacia. (+)-3-^{125}I-MK 801 and ^{35}S-deoxyadenosine 5′-[α-thio] triphosphate were purchased from NEN Dupont. Sequencing was performed using the Sequenase II kit from United States Biochemicals, or through the core laboratory at the Children's Hospital of Philadelphia. Fetal Bovine Serum was a product of Hyclone. Horse Serum, L-glutamine, Penicillin/Streptomycin, and trypsin were from GIBCO BRL. All other reagents used were of the highest quality available from standard commercial sources.

2.2. Cell culture and transfections

Human Embryonic Kidney (HEK) 293t cells were obtained from ATCC with permission of Dr. Michele Calos (Stanford University) and were propagated as previously described for HEK 293 cells (Lynch et al., 1995, Gallagher et al., 1996). Cells were transfected with 1:1 ratios of NR1a and either wildtype, chimeric, or mutant NR2 subunits by calcium phosphate precipitation (Chen and Okayama, 1987). Ten micromolar MK 801 was included during all steps of the transfection to protect against NMDA receptor mediated cell death (Lynch et al., 1995).

2.3. Tissue preparation

Tissue was stored and prepared for assays as described previously (Lynch and Gallagher, 1996). Neonatal brain was obtained from rats in the first 12 h after birth.

2.4. Chimeric NR2 subunit construction and site directed mutagenesis

All mutations and chimeras were produced by overlapping polymerase chain reaction (PCR) with oligonucleotides containing restriction sites within the ε1and/or ε2 receptors. The constructions of the ε1–ε2 chimeras CH1, CH5, CH6, CH8, CH25, CH48, CH58, CH84 (Gallagher et al., 1996), CH9 and CH10 (Gallagher et al., 1998) were previously described. These chimeric receptors contained ε2 sequence in the following regions: CH1-amino acids 138-464; CH5-amino acids 198-1481; CH6-amino acids 198-464; CH8-amino acids 1-464; CH9-amino acids 138-281; CH10-amino acids 198-281; CH25-amino acids 464-1481; CH48-amino acids 1-356; CH58 amino acids 198-356; CH84-amino acids 356-464.

The synthesis of the ε2 mutants ε2E201R, ε2E201N, ε2E201A, ε2E201D, ε2E200A, ε2EE200-201QN and ε1MQN199-201LEE were described in our previous studies (Gallagher et al., 1996, 1997). The mutant ε2EE200-201RR was made using an identical strategy to ε2EE200-201QN but replacing glutamine and asparagine with sequential arginines. The mutant ε2E200Q was made using an identical strategy to ε2E200A but replacing alanine with glutamine.

To assess whether placement of arginine 337 (ε2R337) at the homologous location in ε1 (Q336) increases affinity for ifenprodil, site directed mutagenesis of ε1 was performed using two PCR reactions (Gallagher et al., 1996):

PCR 1: 5′ oligonucleotide (Q336RE15): CCGCTACACACTCTGCACCGGTTTATGGTCAATGTGACTTGG
3′ oligonucleotide: ε1AFL2
PCR 2: 5′ oligonucleotide: sp6
3′oligonucleotide: (Q336RE13)CCAAGTCACATTGACCATAAACCGGTGCAGAGTGTGTAGCGG.

The products from these PCR reactions were digested with Age I and ligated together. This product was used as the template for a PCR reaction using the sp6 and ε1AFL2 primers. This product was then digested with Sal I and Afl

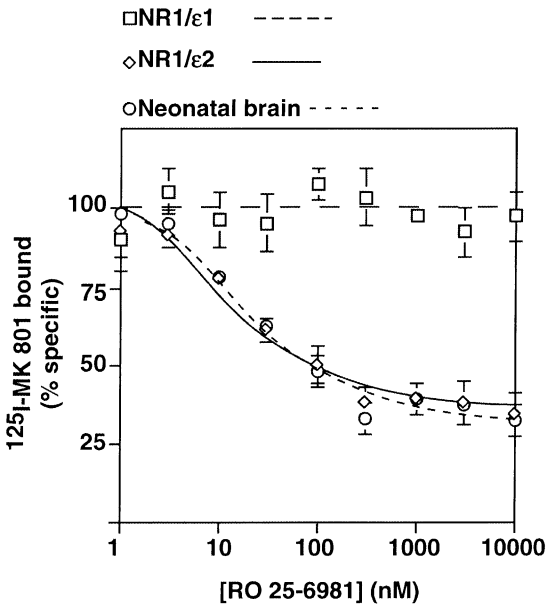

Fig. 1. Selectivity of RO 25-6981 in ligand-binding assays. To confirm that RO 25-6981 modulates ^{125}I-MK 801 binding similarly to its actions in other assays, we investigated the selectivity of this drug for different NMDA receptor subtypes expressed in HEK 293t cells. RO 25-6981 was very potent at receptors containing NR1a/ε2 (IC_{50} value = 18 nM) and at neonatal receptors, while it had no effect on binding to NR1a/ε1 at concentrations as high as 10 μM. The I_{max} value for NR1a/ε2 receptors was 64%. Values shown were mean ± S.E.M. of three to six separate experiments.

II and ligated into ε1 between the Sal I and Afl II sites. All constructs were sequenced for verification.

2.5. Ligand-binding assays

^{125}I-MK 801 binding was performed as described previously (Williams et al., 1993; Lynch et al., 1994). All results were averaged from three to six trials performed in triplicate or duplicate. For routine assays, assays contained 100 μM spermidine and 100 μM magnesium chloride. For assays testing the effect of spermidine, neither spermidine nor magnesium was included in preincubation washes. Non-specific binding was determined by incubation with 10 μM MK801. Typical non-specific binding was 10–15 fmol/mg protein, and displaceable binding to untransfected cells was less than 25% over non-specific levels (Lynch et al., 1994; data not shown). Using these assay conditions, assays appear to reach equilibrium with native receptors, and no significant binding was observed to homomeric NR1a receptors over untransfected cells (Lynch et al., 1994, 1995). For all assays, data were analyzed as inhibition curves using computer software derived from the NIH sponsored PROPHET system to determine best fit IC_{50} and I_{max} (maximal level of inhibition) values. IC_{50} values were log-converted for comparison with wildtype values. The values from mutant receptors were compared statistically to wildtype receptors using ANOVA, followed by Student's *t* test using the InStat computer software for MacIntosh.

2.6. Agonist stimulated Ca^{2+} responses

HEK 293 cells were grown on 35-mm glass bottom dishes (Mattek, Ashland, MA), transfected with NMDA subunit combinations, and assessed using Ca^{2+} imaging on a Nikon microscope in conjunction with Metafluor imaging software (Universal imaging). Twenty-four hours after transfection, cells were rinsed with HEPES buffered saline solution (140 mM sodium chloride, 5.0 mM potassium chloride, 9 mM sodium phosphate, 5.5 mM dextrose, 2 mM calcium chloride, in 20 mM HEPES, pH 7.2) and loaded with 1 μM Fura 2-AM (acetyl–methyl ester). Following 30 min of loading cells were rinsed and background fluorescence levels determined. Agonists were applied (20 μM glutamate, 100 μM glycine) and images collected. Baseline and peak intracellular 340:380 ratios were determined and converted to intracellular Ca^{2+} levels by a calibration curve (Grynkiewicz et al., 1985) (R_{min} = 0.22, R_{max} = 5; K_d = 135 nM; Sf2 = 870; Sb2 = 130). For studies assessing inhibitors, cells were preincubated with inhibitor during the loading of Fura-2AM.

3. Results

3.1. Selectivity of RO 25-6981

To confirm that RO 25-6981 modulated ^{125}I-MK 801 binding similarly to its actions in other assays, we investi-

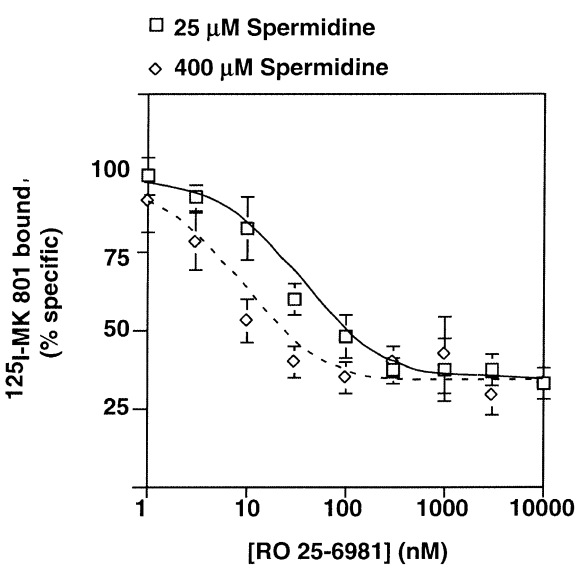

Fig. 2. Effect of changing spermidine concentration on inhibition by RO 25-6981. ^{125}I-MK 801 binding was performed to neonatal brain membranes at multiple spermidine concentrations. Raising the spermidine concentration increased the affinity of RO 25-6981 without changing the I_{max} value. Each point shown was the mean ± S.E.M. of four separate experiments.

gated the selectivity of this drug for neonatal brain and for different NMDA receptor subtypes. RO 25-6981 inhibited ^{125}I-MK 801 binding to neonatal brain with an IC_{50} value of 13 nM and an I_{max} value of 70% (Fig. 1). In recombinant receptors, RO 25-6981 was very potent at receptors containing NR1a/ε2 (IC_{50} value = 18 nM), while it had no effect on binding to NR1a/ε1 at concentrations as high as 10 μM. The I_{max} value for NR1a/ε2 receptors was 64%, consistent with allosteric inhibition. The similarity between neonatal receptors and NR1a/ε2 is consistent with the high levels of the ε2 subunit made in the neonatal period (Zhong et al., 1994).

3.2. Effects of spermidine on RO 25-6981 inhibition

Since spermidine is proposed to act in an overlapping manner with RO 25-6981 and has distinct effects on the actions of haloperidol and ifenprodil in ^{125}I-MK801 binding assays, we sought to determine whether the spermidine concentration in the assay altered the effects of RO 25-6981 in a manner similar to that observed with haloperidol or ifenprodil. When spermidine concentration was increased from 25 to 400 μM, no significant difference was seen in the I_{max} value for inhibition of binding to native neonatal receptors by RO 25-6981 (Fig. 2). This contrasted with the decrease in I_{max} value seen for haloperidol as spermidine concentration was increased (Lynch and Gallagher, 1996). In addition, there was a small decrease in the IC_{50} value (from 25 to 9 nM) for RO 25-6981 which marginally reached statistical significance ($p = 0.05$). This was the opposite result seen from the interaction of ifenprodil with spermidine (Reynolds and Miller, 1989; Dana et al., 1991). Thus, this approach differentiated the actions of RO 25-6981 from those of both haloperidol and ifenprodil.

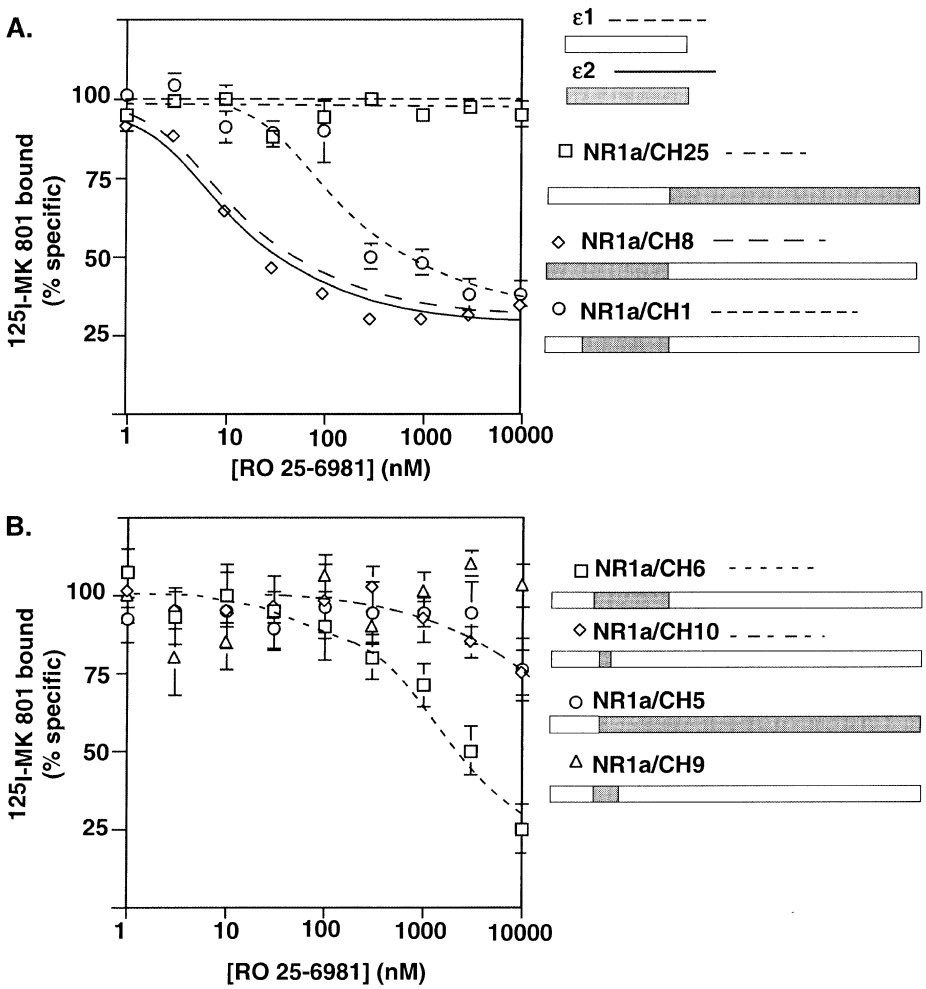

Fig. 3. Inhibition of ^{125}I-MK801 binding to chimeric ε1/ε2 receptors. Inhibition of binding to chimeric receptors was used to localize the sites mediating the effects of RO 25-6981. Chimeras are diagrammed to show the portions containing ε1 (white region) or ε2 (gray region). In panel A, chimeras containing the N-terminal region of ε2 contained the structural determinants for effects of RO 25-6891. Almost complete affinity was retained when the chimera contained ε2 from amino acids 138-464. However, all chimeras with smaller components of ε2 were of lower affinity or efficacy than wildtype (Panels B and C). A few chimeras with small amounts of ε2 (CH 5, CH6, CH10) had some affinity for RO 25-6981 above that seen with ε1. Each point shown was the mean ± S.E.M. of three to four separate experiments. The relative level of binding for different chimeras when expressed with NR1a is shown in Panel D.

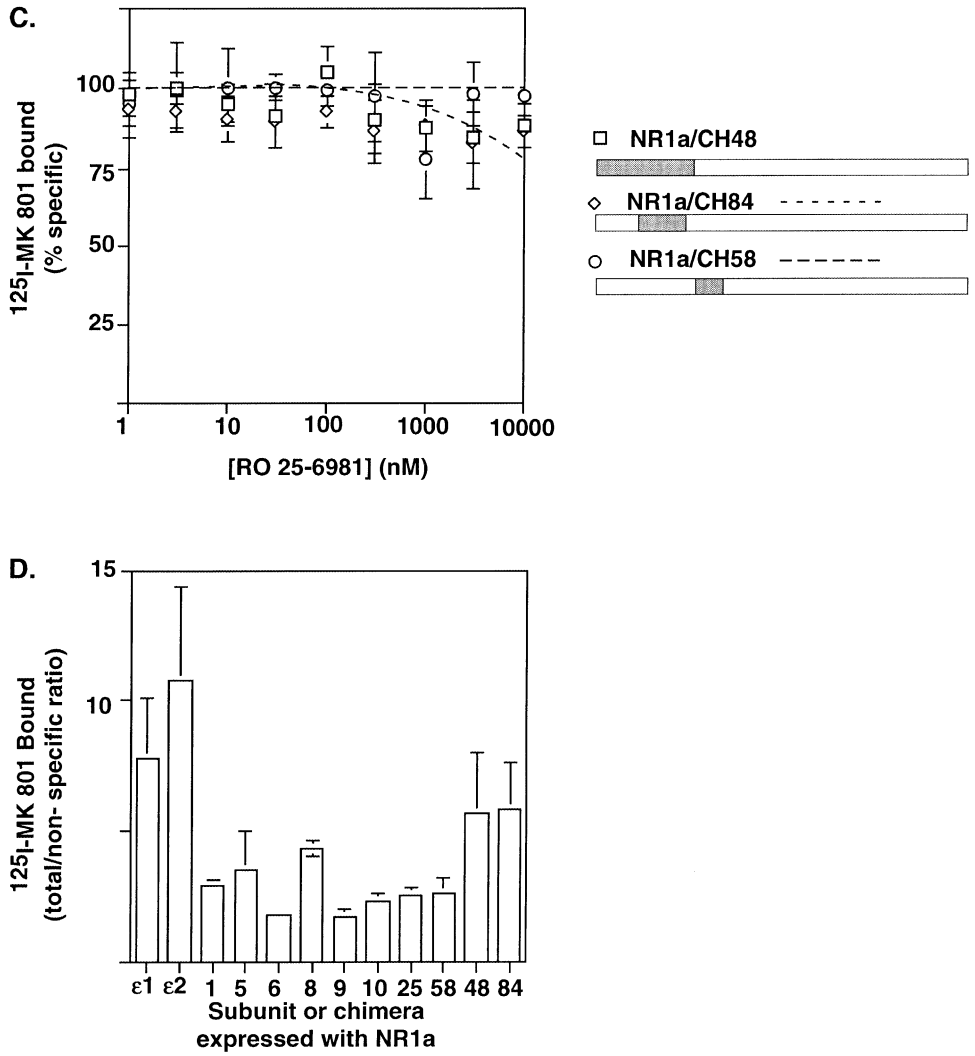

Fig. 3 (*continued*).

3.3. Structural determinants of the effects of RO 25-6981

Previously, we constructed a series of chimeric (CH) NR2 subunits from $\varepsilon 1$ and $\varepsilon 2$ that selectively localize structural determinants for the effects of haloperidol, spermidine, and ifenprodil. We expressed those chimeras with NR1a to assess the components of NR2B ($\varepsilon 2$) which are required for the effects of RO 25-6981. Although the level of binding was decreased from wildtype in most chimeras and varied among the chimeras, specific binding was observed with each chimeric receptor when coexpressed with NR1a (Fig. 3). The structural determinants for RO 25-6981 localized to the N-terminal portion of the molecule, as found previously for haloperidol, ifenprodil and spermidine. Receptors formed from NR1a/CH8 were inhibited by RO 25-6981 with an IC_{50} value (14 nM) and an I_{max} value ($72 \pm 5\%$), similar to those of wildtype $\varepsilon 2$ (Fig. 3). Binding to the inverse chimera (CH25) was not affected by RO 25-6981 at concentrations of up to 10 μM. This demonstrated that all structural determinants for effects of RO 25-6981 that differ between $\varepsilon 1$ and $\varepsilon 2$ were present in the first 464 amino acids of $\varepsilon 2$. We then decreased the amount of $\varepsilon 2$ present in the chimeras to determine structural portions needed for mediation of the effects of RO 25-6981. CH1, which contained $\varepsilon 2$ sequence from amino acid 138-464, had almost the same affinity for RO 25-6918 as wild type $\varepsilon 2$ receptors, and had a similar I_{max} value to wildtype receptors (Fig. 3A). Thus, many of the subunit-specific structural determinants were present in this segment of $\varepsilon 2$. However, unlike haloperidol or ifenprodil, RO 25-6981 had minimal ability to inhibit binding to chimeric NMDA receptors containing smaller amounts of $\varepsilon 2$ (Fig. 3B,C; Table 1). Binding to a few chimeras such as CH10, CH5, and CH6 (co-expressed with NR1a) was inhibited slightly by RO 25-6981, but the IC_{50} values were substantially different from wildtype $\varepsilon 2$ receptor and close to $\varepsilon 1$. Thus, while the localization of structural determinants for RO 25-6981 generally matched that seen for ifenprodil in its localization to the N-terminal region, the structural determinants required for the high

Table 1
Inhibition of wildtype and Chimeric $\varepsilon1/\varepsilon2$ NMDA receptors by RO 25-6981

Receptor	IC_{50} value (μM)
NR1a/ε1	no inhibition
NR1a/ε2	0.018 (0.013–0.024)
NR1a/CH8	0.014 (0.013–0.015)
NR1a/CH1	0.29 (0.19–0.42)[a]
NR1a/CH25	no inhibition
NR1a/CH6	1.6 (0.98–2.7)[b]
NR1a/CH5	8.7 (5.7–13.0)[c]
NR1a/CH9	no inhibition
NR1a/CH10	0.96 (0.22–4.5)[b]
NR1a/CH48	no inhibition
NR1a/CH84	5.1 (2.0–13.1)[c]
NR1a/CH58	no inhibition

Wildtype or chimeric mouse epsilon subunits were transfected with NR1a and inhibition of ^{125}I-MK 801 binding by RO 25-6981 was assessed. Results shown are mean of three to six separate experiments with the values for mean $\pm$ 1 S.E.M. given in parentheses. For the entire group of chimeras, $P < 0.0001$ by ANOVA. No significant differences were seen in I_{max} values.
[a] $P < 0.005$ vs. NR1a/ε2 by *t*-test.
[b] $P < 0.02$ vs. NR1a/ε2 by *t*-test.
[c] $P < 0.005$ vs. NR1a/ε2 by *t*-test.

affinity of RO 25-6981 were spread throughout the N-terminus to a greater degree than for ifenprodil.

3.4. Site directed mutant receptors: ε2R337

In our previous study, chimeric receptors suggested that a specific structural determinant for ifenprodil was present between amino acids 198 and 356, perhaps at ε2R337 (Gallagher et al., 1996). Unfortunately, mutations of this site yielded receptors with very low B_{max} values ($< 5\%$ of wildtype in HEK293t cells) which formed no functional channels in transfected Chinese hamster ovary (CHO) cells, leaving the significance of this residue unclear (Brimecombe et al., 1998; Gallagher et al., 1996). To assess the importance of this residue in a complementary way, we introduced this residue into the homologous segment of ε1 by creating the mutant ε1Q336R. When co-expressed with NR1a, the mutant ε1Q336R possessed a slightly higher affinity for ifenprodil than wildtype ε1 receptors (17 vs. 48 μM) (Table 2; Fig. 4B). In addition, introduction of this mutation into ε1 produced a receptor that was sensitive to RO 25-6981, although the I_{max} value was substantially less than for wildtype ε2 (34%) (Fig. 4A). The mean IC_{50} value was 69 nM. In contrast, the affinity of haloperidol for this receptor was identical to wildtype NR1a/ε1 receptors (Fig. 4C), and NR1a/ε1Q336R receptors were not potentiated by spermidine (Fig. 4D). However, in functional assays examining the effect of ifenprodil and RO 25-6981 on agonist-induced intracellular Ca^{2+} responses, 5 μM RO 25-6981 and 5 μM ifenprodil potentiated NR1a/ε1Q336R receptors moderately (Fig. 4E). Receptors made from NR1a/ε1 were uninhibited at this concentration of ifenprodil or RO 25-6981, while receptors made from NR1a/ε2 were substantially inhibited by both agents. This suggests that ε2R337 may play a role in the effects of ifenprodil and RO 25-6981 in ligand binding assays for ^{125}I-MK 801, but does not mediate inhibition in functional assays.

Table 2
Inhibition of ^{125}I-MK 801 binding to recombinant mutant ε1 and ε2 subunits by RO 25-6981 and ifenprodil

Receptor	IC_{50} value RO 25-6981 (μM)	I_{max} (% total)	IC_{50} value for ifenprodil (μM)
NR1a/ε1	no inhibition	–	48 (43–54)
NR1a/ε2	0.018 (0.013–0.024)	64 $\pm$ 7	0.46 (0.3–1.4) (HA)
NR1a/ε1Q336R	0.069 (0.035–0.141)	34 $\pm$ 9[a]	17 (15–19)[b]
NR1a/ε2E201A	0.021 (0.015–0.032)	78 $\pm$ 5	0.78 $\pm$ 0.3 (HA)[c]
NR1a/ε2E201N	0.008 (0.006–0.012)	83 $\pm$ 3	NA
NR1a/ε2E201D	0.018 (0.011–0.030)	81 $\pm$ 1	0.63 $\pm$ 0.2 (HA)[c]
NR1a/ε2E201R	0.071 (0.050–0.10)[d]	62 $\pm$ 19	0.59 $\pm$ 0.3 (HA)[c]
ε2EE200-201RR	no inhibition	–	28 (20–37)
ε2E200A	0.027 (0.015–0.045)	70 $\pm$ 16	0.38 (0.36–0.40)
ε1MQN199-201LEE	no inhibition	–	NA
ε2E200Q	no inhibition	–	14 (2.1–72)
ε2EE200-201QN	0.054 (0.050–0.058)[d]	81 $\pm$ 5	0.72 (0.56–0.93) (HA)

Wildtype or mutant mouse epsilon subunits were transfected with NR1a, and inhibition of ^{125}I-MK 801 binding by RO 25-6981 or ifenprodil was assessed. Mutation of ε1Q336, ε2E200, or ε2E201 influenced the ability of RO 25-6981 and ifenprodil to inhibit ^{125}I-MK 801 binding. HA = high affinity site. Results shown were mean $\pm$ S.E.M. of three to six separate experiments for I_{max} values. Results for IC_{50} values were shown as mean of three to six separate experiments with 1 S.E.M. above and below the mean given in parentheses. For the entire group of mutants, $P = 0.025$ by ANOVA for IC_{50} values for RO 25-6981, $P = 0.026$ by ANOVA for I_{max} values for RO 25-6981, and $P = 0.0008$ by ANOVA for IC_{50} values for ifenprodil. For individual mutants, NA = not assessed.
[a] $P < 0.005$ vs. NR1a/ε2 by *t*-test.
[b] $P < 0.005$ vs. ε1 by *t*-test.
[c] Indicates data cited from Gallagher et al., 1998.
[d] $P < 0.05$ vs. NR1a/ε2 by *t*-test.

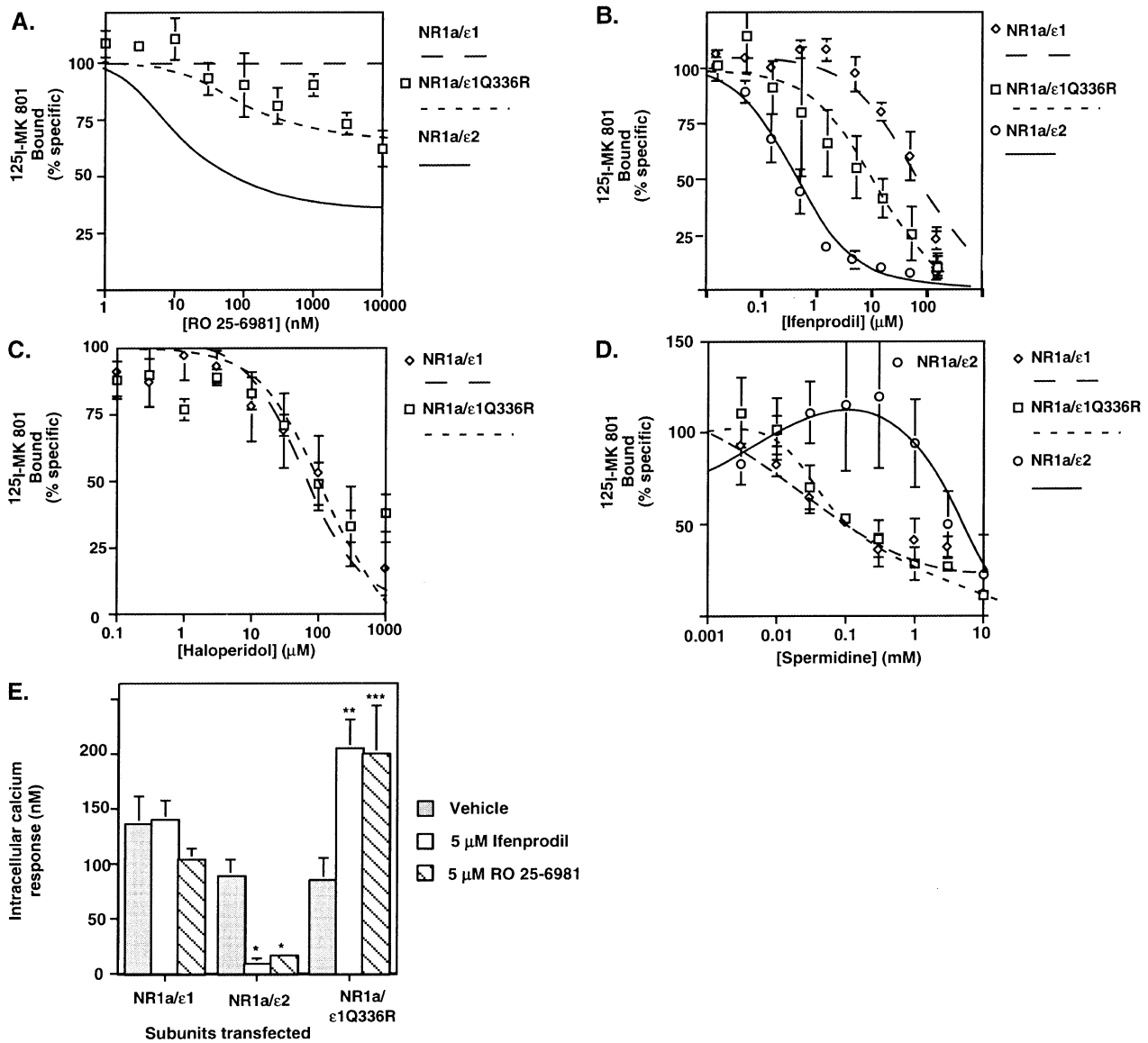

Fig. 4. ε1Q336R containing receptors are slightly inhibited by RO 25-6981. To assess whether ε2R337 was sufficient for effects of RO 25-6981 and related agents, we introduced this residue into the homologous segment of ε1. The introduction of this residue into ε1 with the mutation ε1Q336R produced a receptor which is sensitive to RO 25-6981, although the I_{max} value is low (Panel A). The IC_{50} value was 69 nM. This mutant also demonstrated a threefold higher affinity for ifenprodil than wildtype ε1 receptors (Panel B). In contrast, the affinity of haloperidol was not altered by this mutation, and ε1Q336R receptors were not potentiated by spermidine (Panels C,D). However, RO 25-6981 and ifenprodil potentiated ε1Q336R receptors modestly in functional assays of agonist stimulated Ca^{2+} responses (Panel E). Each point shown was the mean ± S.E.M. of three to four separate experiments for RO 25-6981, haloperidol, ifenprodil and spermidine (Panels A–D). Ca^{2+} imaging experiments represent data from at least three cells from at least two plates for each treatment. $^{*}P < 0.001$, $^{**}P = 0.003$, $^{***}P = 0.02$.

3.5. ε2E200 / E201 mutations

In previous studies, we noted that mutation of ε2E200 and ε2E201 altered haloperidol sensitivity while mutation of ε2E201 removed spermidine stimulation of NMDA receptors (Gallagher et al., 1997, 1998). Mutation of ε2E201 also decreased the sensitivity of other ifenprodil-like drugs in electrophysiological assays, but did not clearly change the affinity of the receptor for ifenprodil in ligand binding approaches (Brimecombe et al., 1998). When we examined the site directed mutant receptor ε2E201R in combination with NR1a, the affinity of RO 25-6981 was slightly decreased without changing the I_{max} value for ^{125}I-MK 801 (Fig. 5). Removing spermidine from the assays did not change the IC_{50} or I_{max} values for this mutant receptor (Table 3). This showed that the decrease in affinity of ε2E201R was independent of spermidine effects. When ε2E201 was changed to alanine, glutamine,

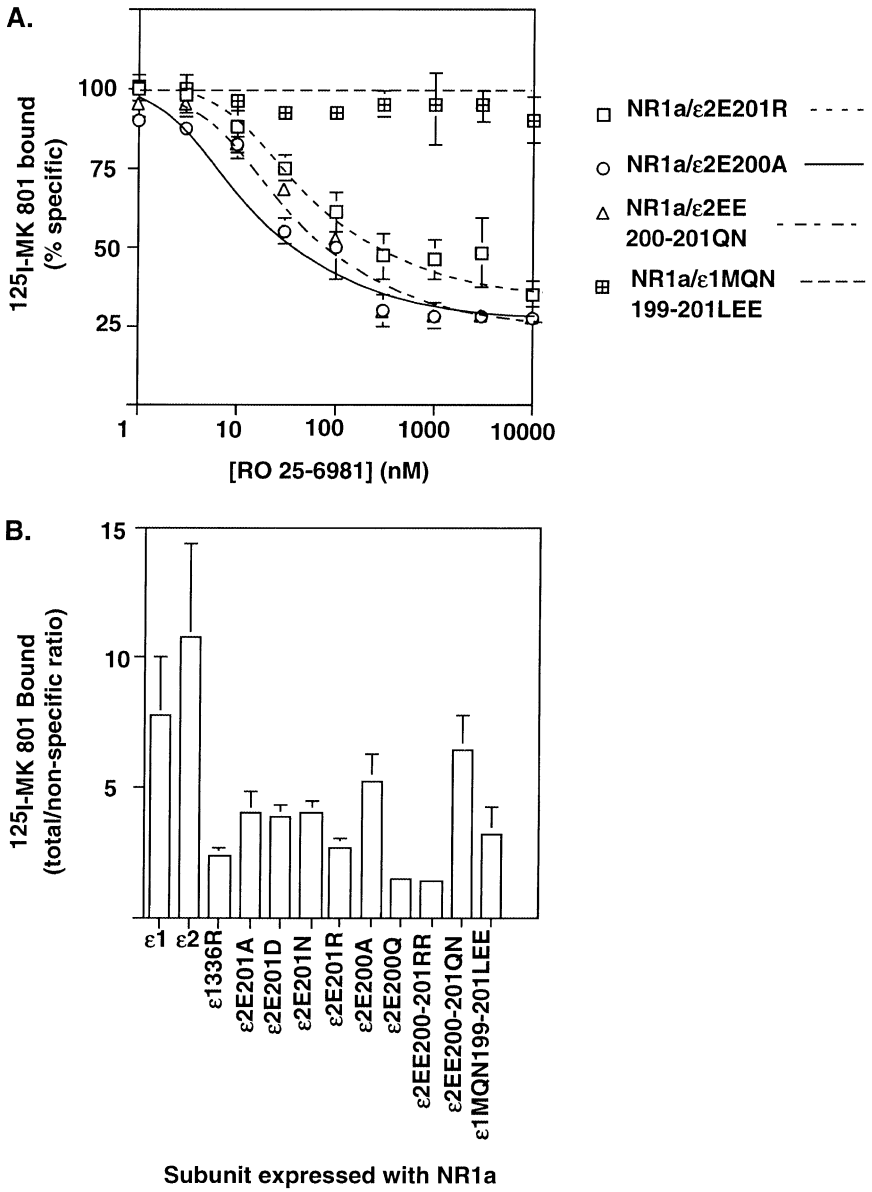

Fig. 5. ε2E200 and ε2E201 control RO 25-6981 effects. Amino acids ε2E200 and ε2E201 were altered to assess their role in the effects of RO 25-6981. When co-transfected with NR1a, receptors containing the mutations ε2E201R or the double mutation ε2EE200-201QN were less sensitive to RO 25-6981 (Panel A). However, the mutation ε2E201A displayed an affinity similar to wildtype ε2. Each point shown is the mean ± S.E.M. of three to four separate experiments. Relative levels of binding for each of these mutants when expressed with NR1a are shown in Panel B.

or asparagine, the IC_{50} values were not different from wildtype ε2. I_{max} values for these receptors were slightly increased, but this did not reach statistical significance.

ε2E200 also appeared to mediate some of the effects of RO 25-6981. When ε2E200 was changed to alanine and coexpressed with NR1a, there was no significant loss in affinity for RO 25-6981 or ifenprodil (Table 2). This mutant retained stimulation by polyamines but was minimally inhibited by haloperidol (Gallagher et al., 1997, 1998). When this residue was changed to glutamine or when ε2E200 and ε2E201 were both changed to arginine, the new receptors were minimally sensitive to RO 25-6981 or ifenprodil. However, the expression levels of the ε2E200Q and ε2EE200-201RR mutants were low based on ^{125}I-MK 801 binding, leaving the significance of these mutations unclear. When amino acids ε2E200 and ε2E201 were both mutated to glutamine and asparagine, respectively (the naturally occurring amino acids in ε1), the resulting receptor had an affinity slightly lower than wildtype ε2 for RO 25-6981 (Fig. 5A, Table 2). These results showed that ε2E200 and ε2E201 both slightly influenced the affinity of RO 25-6981 for modulating NR1a/ε2.

We also sought to determine whether ε2E200 and ε2E201 were sufficient for inhibition by RO 25-6981 (Fig. 5A). When introduced into ε1 with the mutant ε1MQN199-201LEE, the resulting receptor was not inhib-

Table 3
Effect of spermidine concentration on inhibition of binding to NR1a/ε2E201R by RO 25-6981

IC_{50} (nM)	I_{max} (%)
No spermidine	
92 (79–107)	55 ± 12
100 μM spermidine	
114 (88–148)	56 ± 4

Spermidine concentration was varied in ^{125}I-MK 801 binding assays examining the effect of RO 25-6981 binding to NR1a/ε2E201R. No significant difference was seen as a function of spermidine concentration. Results shown were mean ± S.E.M. above and below the mean from four separate experiments.

ited by RO 25-6981 when coexpressed with NR1a, demonstrating that these amino acids alone are not sufficient for mediating the high affinity effects of RO 25-6981.

4. Discussion

The present study demonstrates that specific regions of the ε2 (NR2B) subunit are involved in the modulation of the NMDA receptor by RO 25-6981. Similar to results seen for haloperidol, spermidine and ifenprodil, the actions of RO 25-6981 were governed exclusively by the N-terminal third of the ε2 (NR2B) receptor. In general, the structural components of the NR2B subunit required for effects of RO 25-6981 matched those required for ifenprodil and overlapped with those required for haloperidol and glycine-independent polyamine stimulation (Gallagher et al., 1996, 1997, 1998). However, unlike the results for ifenprodil, chimeric receptors which subdivide the N-terminal region of ε2 between amino acids 138-464 lost their ability to be modulated by RO 25-6981. This suggests that the substantially higher affinity and selectivity of RO 25-6981 requires many specific determinants or substantial tertiary structure in the N-terminal region in order to retain wildtype ability to inhibit ^{125}I-MK 801 binding. The lack of a discrete site for RO 25-6981 binding on NR2B as evidenced by the chimeric receptor studies is most suggestive of an indirect effect by the N-terminal region of NR2B on ifenprodil and RO 25-6081 binding. Masuko et al. (1999) have found that the acidic amino acid NR1D130 and other residues in this region of NR1 are required for high affinity inhibition by ifenprodil in NR1/2B receptors. These residues in NR1 do not appear to interact with polyamines, and may form a binding site for ifenprodil. In previous studies using labeled RO 25-6981 or ifenprodil, autoradiographic distribution of binding sites has matched that of the NR2B subunit in the brain, suggesting direct binding of RO 25-6981 or ifenprodil to the NR2B subunit (Nicolas and Carter, 1994; Mutel et al., 1998). An alternative explanation, more consistent with the findings of Masuko et al. and the present data, is that the NR2B subunit modulates the direct high affinity binding of ifenprodil or RO 25-6981 by the NR1 subunit. This would be analogous to the glycine binding properties of the NMDA receptor. While the glycine-binding site is located on the NR1 subunit, the NR2 subunit present greatly influences the affinity of the receptor for glycine (Honer et al., 1998). These possibilities could be clarified by using direct ligand binding with labeled forms of RO 25-6981. Unfortunately, high levels of binding to untransfected cells have prevented this line of experimentation from achieving success (D.R.L., S.S., unpublished observations).

The present results clarify the overlapping role of amino acids around amino acids ε2E200-201 in the effects of ifenprodil and RO 25-6981. While mutations of ε2E201 did not show decreased sensitivity to ifenprodil in our previous studies assessing effects on ^{125}I-MK 801 binding, such mutations have shown decreased sensitivity to CP101,606 in electrophysiological approaches (Brimecombe et al., 1998). In the present study, the affinity of RO 25-6981 was decreased by some but not all mutations at this site. Thus, there may be a slight difference in the action of ifenprodil on the receptor at this site compared with the higher affinity analogs CP 101,606 and RO 25-6981, but the cumulative results demonstrate that this sequence of acidic amino acids influences the action of all ifenprodil-like drugs. We also considered the possibility that the presence of spermidine, which interacts with ε2E201, could alter the effects of drugs like RO 25-6981 that are mediated through ε2E201. However, changing the concentration of spermidine did not alter the effect of ε2E201 as the mutant ε2E201R had the same affinity for RO 25-6981 in the presence or absence of spermidine. This suggests that the role of these acidic amino acids in the effects of RO 25-6981 does not result merely from competitive antagonism of the effects of spermidine, and that the interactions of these modulators may be complex. In addition, all mutations at ε2E201 (except ε2E201D) diminished polyamine sensitivity, yet only the ε2E201R mutation showed an increase in IC_{50} value for RO 25-6981, suggesting slightly different modulation of RO 25-6981 and spermidine effects by this residue (Gallagher et al., 1997).

Our results suggest that the region around ε2R337 may also influence the effects of ifenprodil and RO 25-6981 in ligand binding assays without influencing the effects of spermidine or haloperidol. The effect we see in receptors containing ε1Q336R is much smaller than the effects demonstrated by Masuko et al., and the effects we note in binding assays are not seen in functional assays (Masuko et al., 1999). This suggests that the role of this region is limited and likely to be indirect. Differences between ligand binding assays and functional assays have been noted for other properties of recombinant NMDA receptors, and suggest the need for parallel evaluation when using these approaches (Monaghan and Larsen, 1997; Chazot et al., 1994; Laurie and Seeburg, 1994). Similarly,

other evidence suggests that ifenprodil may have potentiating effects on native NMDA receptors under some conditions, consistent with the mixed properties it demonstrates at ε1Q336R receptors (Zhang et al., 2000). Differences in mutant receptors between ligand-binding assays and functional assays may represent variation in receptor populations assessed, effects of varying concentrations of modulators such as spermidine, or differences in structural interactions between different modulatory sites on the receptor under different assay conditions.

Our data also demonstrate a pharmacological mechanism for differentiating the actions of RO 25-6981 from those of ifenprodil and haloperidol. The effect of RO 25-6981 is largely unaltered by increasing spermidine concentrations while those of haloperidol and ifenprodil are decreased in differing fashions by spermidine. The IC_{50} value of ifenprodil is increased by increasing spermidine concentration while the I_{max} value of haloperidol is decreased by increasing spermidine concentrations (Reynolds and Miller, 1989; Dana et al., 1991; Lynch and Gallagher, 1996). This could reflect structural actions by spermidine on the N-terminal regions of the NR2B subunit or actions of spermidine within the ion channel itself. As haloperidol and ifenprodil both block the ion channel directly to a small degree, their action may be more susceptible to alteration by the channel blocking actions of spermidine while RO 25-6981, which has essentially no direct effect on the ion channel, is unaffected by spermidine (Ilyin et al., 1996; Legendre and Westbrook, 1991; Fischer et al., 1997). Understanding this effect may be useful in the search for clinically useful compounds retaining the specific mechanism of action used by RO 25-6981.

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