0026-895X/00/030614-00$3.00/0
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 58:614–623, 2000

Vol. 58, No. 3
13087/847054
Printed in U.S.A.

Conantokin G Is an NR2B-Selective Competitive Antagonist of *N*-Methyl-D-aspartate Receptors

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Received November 10, 1999; accepted May 25, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Conantokin G (Con G) is a 17-amino-acid peptide antagonist of *N*-methyl-D-aspartate (NMDA) receptors isolated from the venom of the marine cone snail, *Conus geographus*. The mechanism of action of Con G has not been well defined; both competitive and noncompetitive interactions with the NMDA-binding site have been proposed. In this study the mechanism of action and subunit selectivity of Con G was examined in whole-cell voltage-clamp recordings from cultured neurons and in two electrode voltage-clamp recordings from *Xenopus* oocytes expressing recombinant NMDA receptors. Con G was a potent and selective antagonist of NMDA-evoked currents in murine cortical neurons (IC_{50} = 480 nM). The slow onset of Con G block could be prevented by coapplication with high concentrations of NMDA or of the competitive antagonist (*RS*)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid. Furthermore, in oocytes expressing NR1a/NR2B receptors, Con G produced a rightward shift in the concentration-response curve for NMDA, providing support for a competitive interaction with the NMDA-binding site. Con G produced an apparent noncompetitive shift in the concentration-response curve for spermine potentiation of NMDA responses, but this was due to spermine-induced enhancement of Con G block. Spermine produced a similar enhancement of DL-2-amino-*S*-phosphopentanoic acid block. Finally, Con G selectively blocked NMDA receptors containing the NR2B subunit. These results demonstrate that Con G is a subunit-specific competitive antagonist of NMDA receptors. The unique subunit selectivity profile of Con G may explain its favorable in vivo profile compared with nonselective NMDA antagonists.

Ionotropic glutamate receptors can be divided into three classes on the basis of their functional and pharmacological properties: *N*-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Dingledine et al., 1999). NMDA receptors have received a great deal of interest because of their involvement in synaptic plasticity and neuronal development as well as in the pathogenesis of a variety of neurological disorders, including epilepsy, ischemic cell death, and neurodegeneration. The pharmacology of this receptor is relatively rich and a large number of modulatory sites on the NMDA receptor have been identified that are potential targets for drug discovery. This includes binding sites for the coagonists NMDA and glycine, and the channel pore that is responsible for the voltage-dependent block by Mg^{2+}. In addition, there are allosteric binding sites for endogenous regulatory molecules such as protons, Zn^{2+}, and polyamines, and also pharmacological probes such as ifenprodil and eliprodil (McBain and Mayer, 1994; Dingledine et al., 1999). Although these allosteric agents appear to bind to unique sites on the NMDA receptor, evidence indicates that they share a common effector mechanism.

Cloning studies have identified six cDNAs encoding NMDA receptor subunits: NMDAR1, which has eight splice variants; four NR2 subunits (NR2A–NR2D); and NR3 (McBain and Mayer, 1994; Dingledine et al., 1999). Immunoprecipitation studies indicate that native NMDA receptors are composed of NR1 and one or more NR2 subunits (Chazot et al., 1994; Sheng et al., 1994; Blahos and Wenthold, 1996; Luo et al., 1997). The functional and pharmacological properties of the NMDA receptor are determined by the NMDA receptor subunit composition. For example, polyamine sensitivity is conferred by the presence of the NR2B and the NR1 splice variant lacking exon 5 (Durand et al., 1993; Williams, 1994). Similarly, ifenprodil and other phenylethanolamines show 400-fold selectivity for NR2B-containing receptors but, in contrast to polyamines, do not show NR1 splice variant selectivity (Williams, 1993; Gallagher et al., 1996; Fischer et al., 1997; Mott et al., 1999). Finally, protons are more potent inhibitors at NR1 splice variants that lack exon 5, but show little selectivity for NMDA receptors that contain the NR2A, NR2B, or NR2D subunit (Traynelis et al., 1995; Pahk and

This study was supported by a sponsored research project with Cognetix, Inc., Salt Lake City, UT 84114.

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; Con G, conantokin G; 5,7-DCKA, 5,7-dichlorokynurenic acid; GABA, γ-aminobutyric acid; APV, DL-2-amino-*S*-phosphonopentanoic acid; CPP, (*RS*)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid.

Williams, 1997). Receptors that contain the NR2C subunit are insensitive to protons (Traynelis et al., 1995).

The venom from a variety of species of cone snails contains neuroactive peptides that interact with voltage- and ligand-gated ion channels (Olivera, 1997; McIntosh et al., 1999). One such peptide isolated from the venom of *Conus geographus*, conantokin G (Con G), has been shown to interact with NMDA receptors (Mena et al., 1990). Con G is a 15-amino-acid peptide that is characterized by the presence of four γ-carboxyglutamate residues. Electrophysiological and calcium-imaging studies have demonstrated that Con G is a potent antagonist of NMDA-evoked responses, and these studies suggested that Con G acts competitively at the NMDA recognition site (Hammerland et al., 1992; Haack et al., 1993). Binding studies, in contrast, have shown that Con G has little effect on the binding of the competitive antagonist [^{3}H]CGP-39653 or [^{3}H]5,7-dichlorokynurenic acid (5,7-DCKA) to the NMDA and glycine-binding sites, respectively (Zhou et al., 1996). Rather, these studies have shown that Con G produces a noncompetitive block of spermine-enhanced [^{3}H]MK-801 binding with little effect on glutamate-stimulated or basal [^{3}H]MK-801 binding (Skolnick et al., 1992). On the basis of these studies it has been argued that Con G acts at a unique allosteric modulatory site associated with the polyamine site, distinct from either the NMDA- or glycine-binding sites on the receptor.

To address the existing discrepancy in the mechanism of action, we explored the mechanism of action of Con G in whole-cell voltage-clamp recordings from cultured cortical neurons and in two electrode voltage-clamp recordings from *Xenopus* oocytes expressing recombinant NMDA receptors. In addition, we have examined whether Con G shows subunit-selective effects as has been well documented for other NMDA receptor antagonists such as ifenprodil. Our data indicate that Con G acts as a competitive antagonist of the NMDA-binding site. Furthermore, we show that Con G is a selective antagonist of receptors containing the NR2B subunit with little activity at receptors incorporating the other NR2 subunits. This subunit profile is unique among the competitive antagonists described thus far and may offer therapeutic advantages over other relatively nonselective compounds.

Materials and Methods

Cultured Neuron Electrophysiology

Whole-cell voltage-clamp recordings from cultured cortical neurons were used to examine the effect of Con G on NMDA-evoked currents. Cortical cells were cultured from 15-gestational day-old Swiss-Webster mouse fetuses or 18-day-old Sprague-Dawley rat fetuses and were used 1 to 2 weeks after plating. Recordings were carried out at room temperature (23°C) according to previously described techniques (Donevan and Rogawski, 1996) in a control bathing solution containing 142 mM NaCl, 1.5 mM KCl, 0.1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and 20 mM sucrose (320 mOsm, pH 7.4). The bathing solution also contained 1 μM strychnine to block the glycine receptor and 200 to 500 nM tetrodotoxin to block voltage-gated ion channels. Recordings were obtained with an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA) by using patch electrodes (2–4 MΩ) filled with an intracellular solution containing 153 mM CsCl, 10 mM EGTA, 10 mM HEPES, and 4 mM $MgCl_2$ (290 mOsm, pH 7.4). Currents were filtered at 1 to 2 KHz, digitally sampled at 1 KHz, and acquired on computer with Axotape or pClamp7 software (Axon Instruments). Currents also were recorded on a chart recorder.

Cells were held at −60 mV unless otherwise noted. Agonist and Con G-containing solutions were applied by using a rapid perfusion system that consisted either of a gravity fed multibarreled microperfusion pipette (Donevan and Rogawski, 1996) or a commercially available linear array system (Warner Instruments, Hamden, CT) that was positioned 200 to 400 μm from the cell. NMDA- and non-NMDA-evoked currents were evoked by 10 μM NMDA (in the presence of 1–2 μM glycine) and 100 μM kainate, respectively. Agonists were applied for 2 to 5 s and separated by a 20- to 30-s wash period. With this protocol, ligand-gated currents were relatively stable for the duration of the recording period.

Oocyte Electrophysiology

cDNA Plasmids. NR1A, NR1B, NR2A, NR2B, NR2C, and NR2D were generously provided by Dr. S. Heinemann (Salk Institute, La Jolla, CA) and Dr. P. H. Seeburg (Max Planck Institute for Medical Research, Heidelberg, Germany).

***Xenopus* Oocyte Injections.** Oocytes were removed from *X. laevis* frogs that were anesthetized by immersion in 0.2% tricaine for 15 to 30 min. Harvested ovarian lobes were defolliculated by incubation in 2 mg/ml collagenase (type IA; Sigma, St. Louis, MO) for 2 h at room temperature on an orbital shaker in calcium-free ND-96 solution containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH = 7.6). The oocytes were rinsed five to six times with a Barth's solution that contained 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM HEPES (pH = 7.4), and selected stage V and VI oocytes were stored at 18°C in Barth's solution supplemented with 1 mM sodium-pyruvate, 0.01 mg/ml gentamycin, and an antibiotic-antimycotic solution containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin B (Life Technologies Inc., Gaithersburg, MD).

Oocytes were injected with recombinant receptors 24 h later. Glass capillary tubes (World Precision Instruments, Sarasota, FL) were pulled to a fine tip on a vertical micropipette puller (David Kopf, Tujunga, CA) and broken back or beveled to an outside diameter of 21 μm. RNA stocks were diluted to a final concentration of 1 to 2 μg/μl and injected into oocytes (23–50 nl) with a microinjector (World Precision Instruments).

Electrophysiology. Electrophysiological recordings were performed 3 to 10 days postinjection and were carried out at room temperature in a control Ringers solution containing 115 NaCl, 2.5 KCl, 1.0 $BaCl_2$, and 10 HEPES (pH = 7.4). Two electrode voltage-clamp recordings were obtained with a Geneclamp (Axon Instruments) or Warner oocyte clamp (Warner Instruments, Hamden, CT) amplifier with 3 M KCl-filled microelectrodes (1–5 MΩ). Recordings were carried out at a holding potential of −60 mV unless otherwise noted.

Data Analysis

Concentration effect data were fit to the logistic equation $I_{\text{Con G}} = I/(1 + (IC_{50}/[\text{Con G}])^{n_H})$, where I is the steady-state current evoked by the agonist, $I_{\text{Con G}}$ is the current after steady-state block by Con G, IC_{50} is the concentration of Con G resulting in 50% block, and n_H is an empirical parameter describing the steepness of the curve and has the same meaning as the Hill coefficient. EC_{50} values for NMDA in the absence and presence of different concentrations of Con G were determined from fits to a logistic equation of a similar form. A Schild analysis was performed with these data. The dose ratios were determined as a ratio of the EC_{50} concentration for NMDA in the absence and presence of Con G ($EC_{50\text{control}}/EC_{50[\text{Con G}]}$). A linear regression of log(dose ratio − 1) versus log[antagonist] was performed to estimate the slope and the x-axis intercept (pA_2). Sigmaplot (SSPS, Chicago, IL) was used for nonlinear curve fitting. Data are presented as mean ± S.E.; n is the number of cells tested.

The statistical significance of differences between population means was assessed with a paired or unpaired t test as appropriate.

Results

Con G Is a Potent and Selective Antagonist of NMDA Receptors in Cultured Neurons

Whole-cell recordings were obtained from cultured mouse and rat cortical neurons at 7 to 10 days after plating. NMDA (10 μM), in the presence of 1 μM glycine, was applied for 2 to 5 s at 20- to 30-s intervals and evoked inward current responses (holding potential of −60 mV) that showed minimal rundown during prolonged recordings. As shown in Fig. 1A, incubation in 3 μM Con G-containing buffer produced a slowly developing block of the NMDA response from a whole-cell recording in a mouse cortical neuron that reached steady state within 1 to 2 min. Recovery from block occurred over an even longer time period (>5–10 min) once Con G was removed. Due to the slow recovery from block of the NMDA response by Con G, it was difficult to test a single neuron with more than two concentrations of Con G. Fractional block values from a series of similar experiments with various concentrations of Con G in recordings from mouse cortical neurons are plotted in Fig. 1B. There was a concentration-dependent increase in fractional block as the concentration of Con G was increased from 100 nM to 10 μM. The IC_{50} value for block of the NMDA-evoked current in mouse cortical neurons was 480 nM (n_H = 1). Con G block of NMDA currents in recordings from rat cultured cortical neurons was more variable; block of NMDA responses in rat neurons by 3 μM Con G ranged from 43 to 85% inhibition. Most studies were carried out with mouse cortical cells, unless otherwise noted.

Con G was specific for the NMDA subtype of glutamate receptors. As shown in Fig. 2A and summarized in the plot in Fig. 2B, 10 μM Con G produced almost complete block of NMDA receptor currents, yet had minimal effects on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor responses evoked by 100 μM kainate. In addition, 10 μM Con G had negligible effects on γ-aminobutyric acid $(GABA)_A$ receptors. The 3 μM GABA-evoked current responses after a 2- to 3-min incubation in 10 μM Con G were similar to control (fraction of control: 1.02 ± 0.03, n = 4).

Con G Block Is NR2B Selective

Polyamine, proton, phenylethanolamine, and Zn^{2+} modulation of the NMDA receptor has been shown to be NR2 subunit and/or NR1 splice variant specific (Dingledine et al., 1999). In particular, glycine-independent potentiation of the NMDA receptor by polyamines is specific to receptors containing NR1 splice variants that lack exon 5, and that contain the NR2B subunit (Durand et al., 1993; Williams, 1994). Proton sensitivity, however, is reduced by the presence of the exon 5 in NR1 but is independent of the NR2 subunit expressed (Traynelis et al., 1995). Finally, phenylethanolamines selectively block receptors incorporating the NR2B subunit and show negligible NR1 splice variant selectivity (Williams, 1993; Mott et al., 1999). Given the proposed interaction between Con G and the polyamine site (Skolnick et al., 1992), we examined whether Con G showed NR2 subunit and NR1 splice variant selectivity for block of recombinant NMDA receptors in two electrode voltage-clamp recordings from oocytes injected with different NR1 splice variants and NR2 subunit combinations.

The traces in Fig. 3A are from an oocyte expressing NR1A/NR2B heteromeric receptors. Con G produced a potent block of the NMDA-evoked current response, with an IC_{50} in this

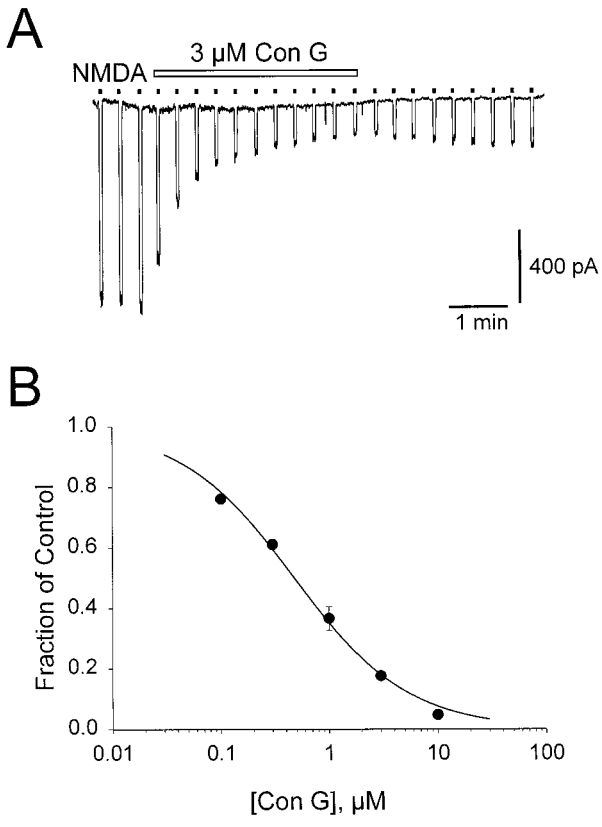

Fig. 1. Con G is a potent antagonist of NMDA-evoked currents in neurons. Current trace in A is a whole-cell voltage-clamp recording from a cultured mouse cortical neuron and shows the slow onset and recovery from Con G block of inward currents (−60 mV) evoked by 10 μM NMDA (+1 μM glycine). Plot in B shows concentration-dependent block of NMDA currents determined in experiments similar to and including that shown in A (n = 4–6 cells at each concentration). Data points in this and subsequent figures are mean ± SE.

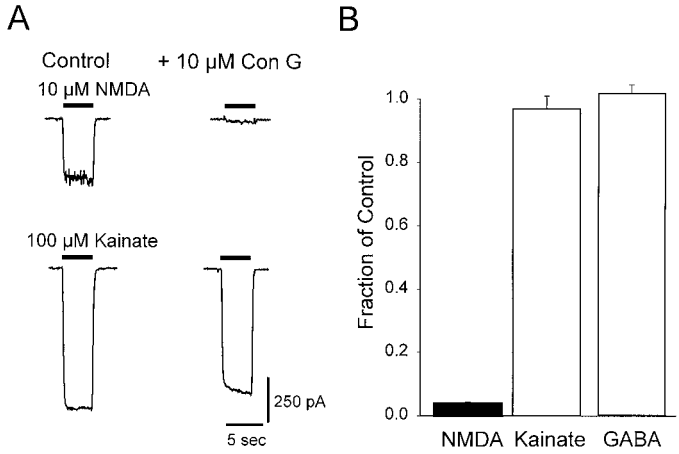

Fig. 2. Con G is selective for NMDA receptors. Traces in A are from the same mouse cortical neuron and show 10 μM NMDA- (top traces) and 100 μM kainate-evoked responses before and after steady-state block of the NMDA response by 10 μM Con G. Data similar to and including those shown in A are summarized in B, which also shows the lack of effect of Con G on 3 μM GABA-evoked currents. n = 4 cells tested with each agonist.

oocyte that was significantly less than 1 μM. The data from this and other oocytes expressing this subunit combination are summarized in Fig. 3B (closed circles). The IC_{50} for Con G block of NR1a/NR2B receptors was 717 $\pm$ 65 nM (n = 6). Other NR1A/NR2 subunit combinations were tested. Con G, at concentrations up to 10 μM, had minimal effects on NMDA responses from NR1A/NR2A, NR1A/NR2C, and NR1A/NR2D subunit combinations. Block of NMDA responses by 3 μM Con G was somewhat greater at NR1B/NR2B (fraction of control: 0.06 $\pm$ 0.01, n = 2) than NR1A/NR2B (fraction of control: 0.15 $\pm$ 0.03, n = 6) subunit combinations. These data indicate that Con G is an NR2B-selective antagonist.

As discussed above the onset and recovery from block of the NMDA-evoked currents in the neuronal recordings was slow. The oocyte preparation showed little rundown of NMDA responses with prolonged application of NMDA and it was possible to more fully characterize the onset and recovery from block of NMDA-evoked current responses by Con G in this system. We examined the onset and recovery from block of 10 μM NMDA (in the presence of 10 μM glycine) currents in oocytes expressing NR1A/NR2B receptors (Fig. 3C). The current trajectories during onset of block by 0.3 and 3 μM Con G were well fit by single exponential functions with a τ_{on} of 8.1 $\pm$ 0.5 and 33.9 $\pm$ 3.4 s, respectively (n = 6). The trajectory of the current response during recovery from 0.3 and 3 μM Con G block was also well fit by a single exponential with τ_{off} of 124.1 $\pm$ 13.8 and 152.8 $\pm$ 10.4 s, respectively (n = 6). These values were not significantly different.

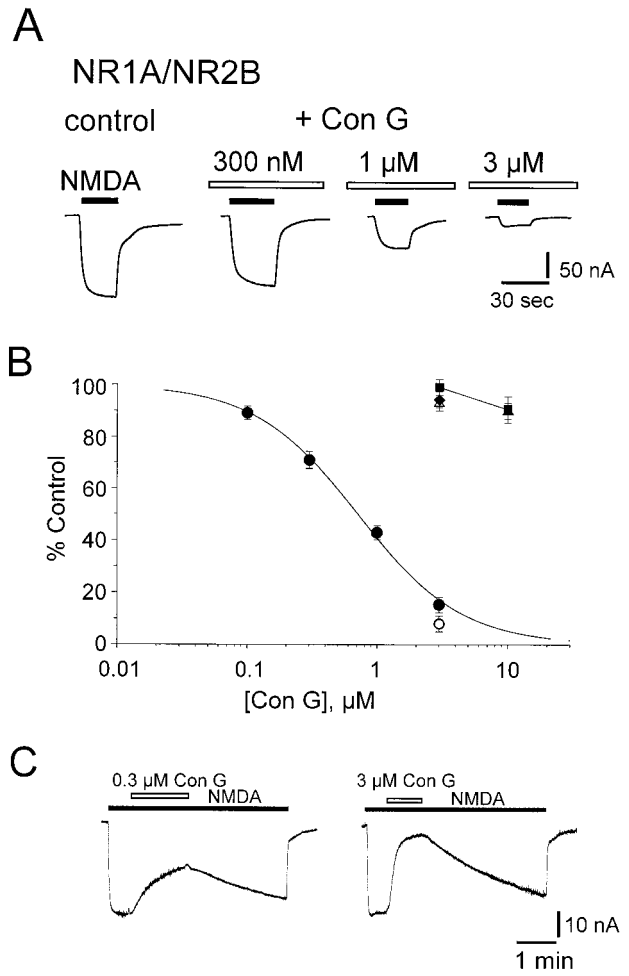

Fig. 3. Con G is selective for the NR2B subunit. Traces in A are from the same oocyte expressing NR1a/NR2B receptors and show the concentration-dependent block of 100 μM NMDA-evoked (+2 μM glycine) currents by Con G. Data similar to and including those shown in A are summarized in B (●). B also plots similar block of NMDA currents in oocytes expressing NR1b/NR2B (○) receptors by 3 μM Con G and lack of effect of 3 to 10 μM Con G on NMDA responses from oocytes expressing NR1a/NR2A (■), NR1a/NR2C (♦), or NR1a/NR2D (△) receptors. n = 3 to 8 oocytes/receptor combination at each concentration tested. Traces in C are from the same oocyte expressing NR1A/NR2B receptors and show the kinetics of block of 10 μM NMDA (+10 μM glycine) responses by 0.3 and 3 μM Con G.

Mechanism of Block

Con G Does Not Bind in the Channel. The relatively slow onset of block of NMDA receptors by Con G is reminiscent of the profile of high-affinity, use-dependent, channel blockers such as MK-801. To examine this possibility more directly we compared Con G block of the NMDA response in the absence and presence of agonist. As shown in the upper trace in Fig. 4A, coapplication of 3 μM Con G with 10 μM NMDA produced a slowly developing block of the NMDA response that reached steady state in approximately 1 min. Application of Con G in the absence of NMDA over the same time period, however, produced a similar block of the NMDA response. In addition, incubation of Con G in the presence of Mg^{2+} (see below) did not prevent block, indicating that channel opening was not required for access of Con G to its binding site. Recovery from block also occurred in the absence of channel activation (data not shown). Thus, the onset and recovery from Con G block of the NMDA response did not show use dependence. Another feature of charged channel blockers is the voltage dependence of block. As shown in Fig. 4B, Con G block of the NMDA response was similar at negative (−60 mV) and positive (+60 mV) holding potentials, indicating a lack of voltage dependence of Con G block. The lack of use and voltage dependence of block suggests that Con G acts a site outside of the ion channel pore.

Competitive Interaction with NMDA-Binding Site. Previous electrophysiological studies have indicated that Con G acts as a competitive antagonist at the glutamate-binding site (Hammerland et al., 1992; Benke et al., 1993; Haack et al., 1993). Given the slow kinetics of Con G block, it would be difficult to distinguish between a competitive antagonist that unbinds very slowly (as shown above) and a noncompetitive antagonist. Thus, it was not possible to carry out classical competition experiments in the neuronal recordings. Instead, we took advantage of the slow kinetics of the Con G block and the rapid kinetics of classical ligands acting at the NMDA recognition site. We examined whether NMDA or the competitive antagonist (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), would compete with Con G for a common binding site and thus occlude Con G block. As described above and shown in the top trace in Fig. 5A, incubation in 3 μM Con G produced almost complete block of the NMDA response; the NMDA response immediately after Con G application was 20.2 $\pm$ 2.3% (n = 8) of the control NMDA response before incubation in Con G. In contrast, coapplication of Con G with high concentrations of NMDA (in the presence of 10 mM Mg^{2+} to prevent channel opening; middle trace) or the competitive antagonist CPP (50 μM; bottom trace) prevented development of block by Con G. The NMDA response after incubation in Con G in the presence of NMDA or CPP was 89.6 $\pm$ 5.4% (n = 4) and 91.0 $\pm$ 4.1% (n = 4) of

the control NMDA response, respectively. In an additional series of experiments 10 mM Mg^{2+} had no effect on Con G block (data not shown). Data from a series of experiments similar to that shown in Fig. 5A are summarized in the plot in Fig. 5B. These data clearly indicate that ligands at the NMDA recognition site prevent access of Con G to its binding site, and provide support for a competitive mechanism of action of Con G. To address this possibility more directly, we examined the effect of Con G on the concentration-response relationship for NMDA-evoked current responses in recordings from oocytes expressing receptors composed of NR1A and NR2B subunits. This system permits prolonged incubation in agonist- and antagonist-containing solutions and thus allows time to reach equilibrium. The graph in Fig. 6A shows the concentration-response curves for NMDA-evoked responses in the absence and presence of 1 μM Con G. In the absence of Con G the potency of NMDA at NR1A/NR2B receptors was 29.6 $\pm$ 2.4 μM (n = 6). In the presence of Con G there was a concentration-dependent rightward shift in the concentration response curve for NMDA, with no significant change in the maximal response to NMDA. A Schild analysis was carried out with these data (under *Materials and Methods*) to confirm the competitive nature of Con G block and determine the potency of Con G at NMDA receptors. The Schild regression (Fig. 6B) was linear with a slope of 1. The pA_2 was 6.39, which corresponds to a K_b of 407 nM. These data are consistent with a competitive interaction between NMDA and Con G for a common binding site.

Similar experiments characterized the interaction of ligands at the glycine recognition site with Con G in whole-cell recordings from rat cortical neurons. As expected, coincubation of 3 μM Con G with high concentrations of glycine had no effect on the magnitude of Con G block (Fig. 7B). In fact, there was a trend toward an enhancement of block in the presence of glycine, although this was not observed in every cell tested. Similar studies were carried out with the potent glycine site antagonist 5,7-DCKA. Rather surprisingly, 50 μM 5,7-DCKA was able to prevent Con G block (data not shown). However, at modest concentrations 5,7-DCKA will displace NMDA site ligand binding (Baron et al., 1992), and it is possible that this could explain our observations. To address this possibility we examined the effects of 5,7-DCKA at lower concentrations. Even at this somewhat lower concentration (10 μM), 5,7-DCKA was still able to relieve Con G block (Fig. 7A, middle traces). Moreover, glycine (100 μM) could reverse the effects of 5,7-DCKA (Fig. 7A, bottom traces), suggesting that the reversal of Con G block by 5,7-DCKA was mediated through an interaction with the glycine site and not the NMDA recognition site. In a separate series of experiments, a similar, prolonged application of 5,7-DCKA in the absence of Con G had no effect on the NMDA response. The NMDA response after a 2-min incubation in 10 μM 5,7-DCKA was identical with the control response preceding 5,7-DCK application (99.1 $\pm$ 0.7% of control, n = 3). We also tested HA-966, a partial agonist at the binding site with negligible activity at the NMDA binding site, and this glycine site ligand had minimal effects on Con G block (Fig. 7B). These observations suggest that Con G does not act at the glycine site. Rather, Con G binding can be modified in an allosteric manner by selected ligands binding to the glycine site (i.e., 5,7-DCKA).

Polyamines Enhance Con G Block. Initial binding studies identified a novel allosteric interaction between Con G and the polyamine binding site, whereby Con G produces a noncompetitive displacement of spermine-enhanced MK-801 binding, with little effect on basal or glutamate-enhanced MK-801 binding (Skolnick et al., 1992). Thus, we examined the effect of spermine on the development of Con G block in whole-cell recordings from cultured rat cortical neurons. In addition, we tested the effect of Con G on the potentiation of NMDA-evoked currents by spermine in two electrode voltage-clamp recordings from oocytes expressing NR1A/NR2B heteromeric receptors.

As shown in sample traces in Fig. 8A and summarized in the plot in Fig. 8B, 300 μM spermine did not prevent block of 10 μM NMDA-evoked currents by 3 μM Con G in whole-cell recordings from rat cultured neurons. In fact, there was a significant increase ($P < .001$) in Con G block in the presence of spermine compared with Con G block in the absence of spermine (see below). The NMDA response after incubation

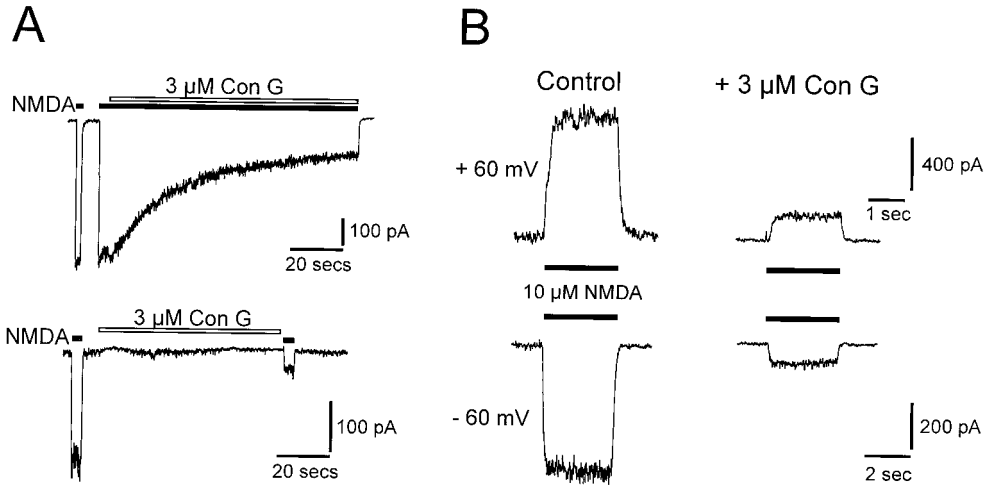

Fig. 4. Con G is not a channel blocker. Traces in A are from two different mouse cortical neurons and demonstrate the slow onset of Con G block of the 10 μM NMDA-evoked current that occurs in the presence (top trace) and absence (bottom trace) of agonist, indicating block is not use-dependent. Traces in B are recordings from two different neurons and show the NMDA-evoked current response before (left) and after (right) steady-state block by 3 μM Con G at hyperpolarized (−60 mV, bottom traces) and depolarized (+60 mV, top traces) holding potentials. Con G block does not show voltage dependence.

in Con G was 42.1 ± 2.6% (n = 4) and 24.1 ± 1.4% (n = 4) of the control NMDA response in the absence and presence of spermine, respectively. This enhancement in block is reminiscent of binding studies demonstrating a spermine-induced enhancement of [^{3}H]CGP39653 binding (Reynolds, 1994). Moreover, electrophysiological studies with NR1A/NR2B-expressing oocytes demonstrating that spermine reduces glutamate potency that would thus increase the apparent affinity of competitive antagonists such as Con G (Williams, 1994). Finally, Hollmann et al. (1993) have demonstrated that inclusion of exon 5, which behaves like spermine (Traynelis et al., 1995), in NR1 reduces the potency of glutamate and increases the potency of the competitive antagonist DL-2-amino-*S*-phosphonopentanoic acid (APV). Consistent with this, Con G was somewhat more potent at receptors containing the exon 5 insert (Fig. 3B), although this was not explored in detail. Thus, spermine does not appear to prevent or occlude access of Con G to its binding site on the NMDA-receptor complex.

To address whether Con G directly or indirectly regulates spermine binding (at least from a functional level) we examined the effect of spermine on NMDA-evoked currents in the absence and presence of Con G. These studies were carried out in voltage-clamp recordings from oocytes expressing NR1A/NR2B heteromeric receptors because glycine-independent enhancement of NMDA responses by spermine has been shown to be dependent on the presence of these two subunits (Durand et al., 1993; Williams, 1994). To examine the effects of Con G on spermine potentiation we determined the concentration dependence of spermine potentiation of NMDA-evoked responses in the absence and presence of Con G. Figure 9A illustrates recordings from a single oocyte and shows the enhancement of the inward current evoked by 100 μM NMDA by 300 μM spermine in control (left) and after steady-state block of the NMDA response by 1 μM Con G (right). Spermine produced an enhancement of the NMDA response and this enhancement was reduced in the presence of Con G. The graph in Fig. 9B demonstrates that Con G produced a noncompetitive-like reduction in the concentration-dependent potentiation of NMDA responses by spermine. These data would support previous binding studies showing that Con G produces a noncompetitive inhibition of spermine-enhanced MK-801 binding. However, a similar shift could be produced if spermine enhanced Con G block. For instance, previous studies have shown that spermine reduces glutamate affinity, which would thus enhance the apparent affinity of competitive antagonists such as Con G (Williams, 1994). As shown in the inset in Fig. 9A, the shape of the spermine-enhanced NMDA response was very different in the presence than in the absence of Con G. There was a slow reduction in the spermine-potentiated NMDA current in the presence of Con G that was not observed in the absence of Con G as if spermine was enhancing Con G block of the NMDA response. To address this possibility more directly, we examined Con G block in the absence and presence of spermine in oocytes expressing NR1a/NR2B receptors. The left trace in Fig. 9C shows minimal block of the NMDA-evoked current response by 1 μM Con G. The trace on the right is from the same oocyte and shows that in the presence of 300 μM spermine, the same concentration of Con G produces a much greater block of the NMDA current. Data from this and several other oocytes are summarized in the plot in Fig. 9D and clearly demonstrate that spermine produces a concentration-dependent enhancement of Con G block. Similar studies were carried out with the competitive antagonist APV and, as was observed with Con G, APV block of NMDA

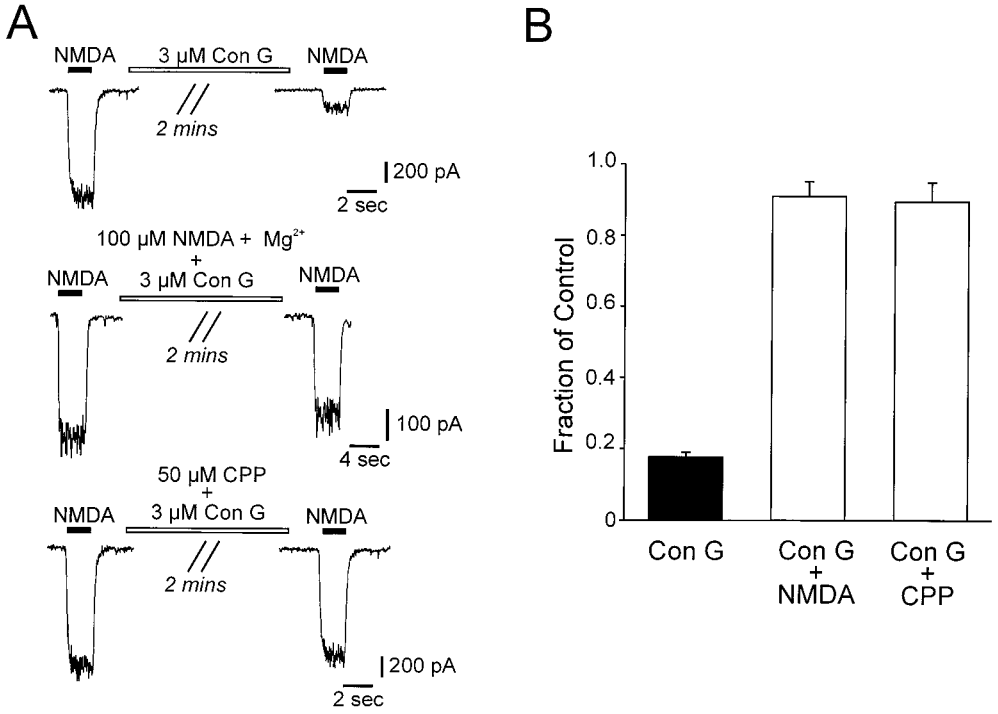

Fig. 5. NMDA ligands prevent Con G block. Traces in A are from three different mouse cortical neurons and show that the block of the 10 μM NMDA-evoked current by 3 μM Con G (top trace) can be prevented by coapplication with high concentrations of NMDA (middle trace) or the competitive antagonist APV (bottom trace). Data similar to and including those shown in A are plotted in B. n = 4 to 6 cells/treatment group.

responses was significantly enhanced ($P < .01$) in the presence of spermine. APV (3 μM) produced a 19.9 ± 0.1 and 32.8 ± 1.1% block of the NMDA response in the absence and presence of 300 μM spermine, respectively ($n = 4$). This is consistent with previous binding studies demonstrating that spermine enhances binding affinity of the selective NMDA site antagonist [^{3}H]CGP39653 (Reynolds, 1994), and conversely, electrophysiological studies by Williams (1994) that demonstrated a spermine-induced reduction in glutamate potency. Moreover, these data indicate that the noncompetitive-like inhibition of spermine potentiation of NMDA responses by Con G reflects an effect of spermine on Con G block and not an effect of Con G on spermine potentiation.

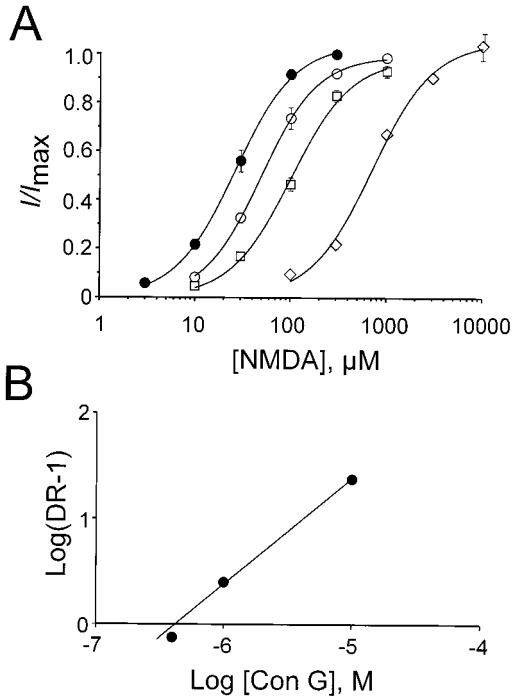

Fig. 6. Con G is a competitive antagonist at the NMDA recognition site. A, concentration dependence for NMDA-evoked currents in oocytes expressing NR1a/NR2B receptors in the absence (control, ●) and presence of 0.3 (○), 1 (□), and 10 μM (◇) Con G. Currents at each concentration were normalized to the 300 μM NMDA response in the absence of Con G. The EC_{50} values for NMDA in control and in the presence of 0.3, 1, and 10 μM Con G were 29.6 ± 2.4 (n_H, 1.4 ± 0.1), 50.1 ± 6.2 (n_H, 1.5 ± 0.1), 103.4 ± 6.3 (n_H, 1.4 ± 0.1), and 730 ± 92 μM (n_H, 1.4 ± 0.1), respectively. $n = 4$ to 8 oocytes at each concentration. B, Schild regression derived from the data in A, where the dose ratio was calculated as the EC_{50} for NMDA in the presence of Con G over the EC_{50} for NMDA in the absence of Con G.

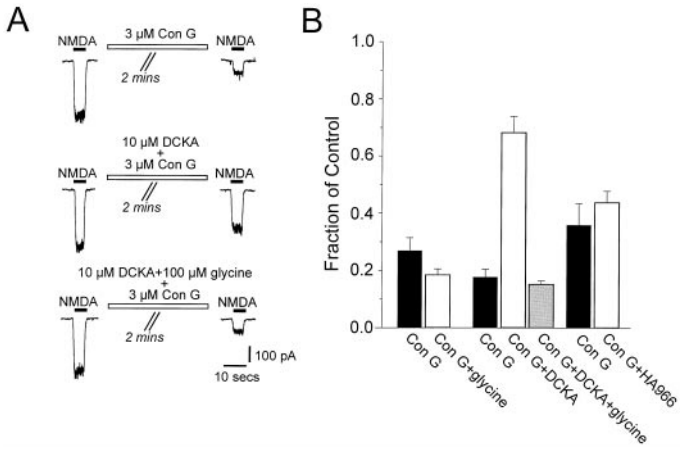

Fig. 7. Allosteric interaction between Con G and glycine site. Traces in A are from the same rat cortical neuron and show the NMDA-evoked current before and after prolonged incubation in Con G in the absence (top traces) or presence of 10 μM 5,7-DCKA (middle traces) or DCKA and 100 μM glycine (bottom traces). Data similar to and including those in A are summarized in the graph in B, which also includes similar experiments in which block by 3 μM Con G was characterized in the absence and then presence of 100 μM glycine or with 300 μM HA-966. $n = 5$ to 8 cells/treatment group. Because of the variability in Con G block in rat cortical neurons each cell was tested with Con G alone and then with Con G and the selected glycine site ligand(s).

Con G block bears some similarity to block of NMDA responses by ifenprodil and ifenprodil-like compounds, in that it shows relatively slow blocking kinetics and as will be described below shares similar NR2 subunit selectivity (Williams, 1993; Kew et al., 1996). Recent studies have suggested that ifenprodil shows a unique agonist- or state-dependent block such that block is more potent and efficacious with high than low concentrations of agonist (Kew et al., 1996). As discussed above and shown in Fig. 4C, Con G does not share this use-dependent mechanism of action. Rather, Con G block occurs in the absence of agonist and block is reduced in the presence of high concentrations of agonist (Fig. 5A, middle trace). These observations indicate that Con G and ifenprodil differ with respect to their mechanism and site of action on the NMDA receptor.

Recent studies by Mott et al. (1999) have demonstrated that ifenprodil and other related phenylethanolamines inhibit NMDA receptors by enhancing proton block. We examined the possibility that Con G interacts with the proton

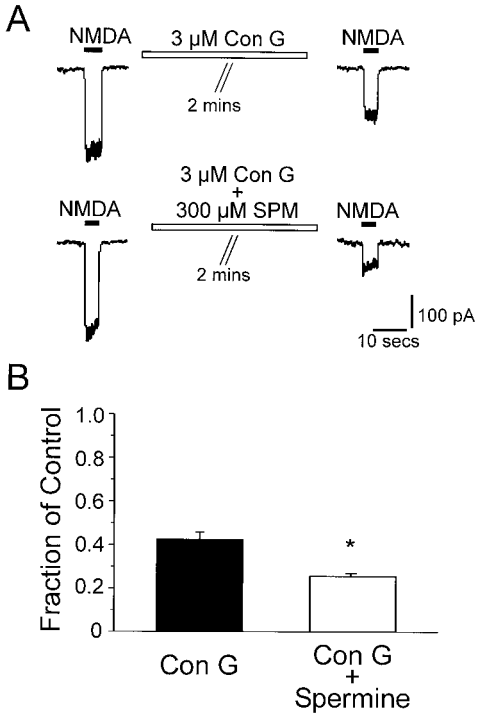

Fig. 8. Spermine does not occlude Con G block. Traces in A are from the same rat cortical neuron and show 10 μM NMDA-evoked currents before and after prolonged incubation in Con G in the absence (top trace) or presence (bottom traces) of 1 mM spermine. Graph in B summarizes data similar to and including those shown in A and shows that Con G block of NMDA responses is not occluded by polyamines. $n = 5$ cells/group. *, significantly different from control ($P < .001$, unpaired t test).

sensor by testing whether Con G block is modified by pH, as has been observed with ifenprodil and CP101,606. As shown in the traces in Fig. 10A, and summarized in the graph in Fig. 10B, pH had no effect on Con G block of NMDA responses in whole-cell recordings from rat cortical neurons. The fractional block of NMDA responses by 1 μM Con G was similar at pHs 8.0, 7.4, and 6.8. These data indicate that Con G does not interact with the proton sensor and thus does not share a common mechanism with ifenprodil and other phenylethanolamines.

Discussion

In this study we examined the mechanism of action of Con G on native and recombinant NMDA receptors in voltage-clamp recordings from cultured cortical neurons and oocytes injected with various NMDA receptor subunit combinations. We found that Con G acts as a competitive antagonist at the NMDA recognition site. Moreover, Con G shows marked selectivity for receptors containing the NR2B subunit, in contrast with other competitive antagonists that do not show such specific subunit selectivity.

Previous studies investigating the mechanism of action of Con G arrived at conflicting conclusions. Initial electrophysiological studies demonstrated that Con G produced a rightward shift in the concentration-response curve for NMDA in oocytes injected with rat brain mRNA (Hammerland et al., 1992; Haack et al., 1993). In addition, subsequent imaging studies showed that Con G produced a similar rightward shift in the concentration-response relationship for NMDA-evoked calcium fluxes (Haack et al., 1993). These two studies provide support for a competitive interaction between Con G and glutamate for the NMDA recognition site. The results from binding studies, however, led to very different conclusions. In these studies, Con G had minimal effect on basal or glutamate- and glycine-enhanced [^{3}H]MK-801 binding. Rather, Con G produced a noncompetitive-like inhibition of spermine-enhanced [^{3}H]MK-801 binding (Skolnick et al., 1992). From these latter studies it was concluded that the mechanism of action of Con G block of NMDA responses was through a novel allosteric modulation of polyamine binding. However, the electrophysiological and functional studies demonstrating Con G block of NMDA responses were carried out in the absence of spermine. Thus, if modulation of polyamine binding was responsible for the action of Con G then Con G should have little effect in these conditions. Our own data would indicate that Con G interacts in a competitive manner with the NMDA-binding site. Thus, ligands that bind to the NMDA recognition site when coapplied with Con G were able to prevent the long-lasting block of NMDA-evoked currents. Moreover, in two electrode voltage-clamp recordings from oocytes expressing NR1A/NR2B containing NMDA receptors, Con G produced a competitive-like rightward shift in the concentration-response curve for NMDA. Glycine, however, did not occlude binding of Con G. Rather, there was a trend toward an increase in Con G block in the presence of glycine. Surprisingly, the competitive glycine site antagonist 5,7-DCKA was able to prevent Con G block. The effects of 5,7-DCKA could be reversed by glycine, suggesting that 5,7-DCKA reduces Con G block through an allosteric interaction between the glycine- and glutamate-binding sites. There is an abundance of literature supporting such an interaction (McBain and Mayer, 1994).

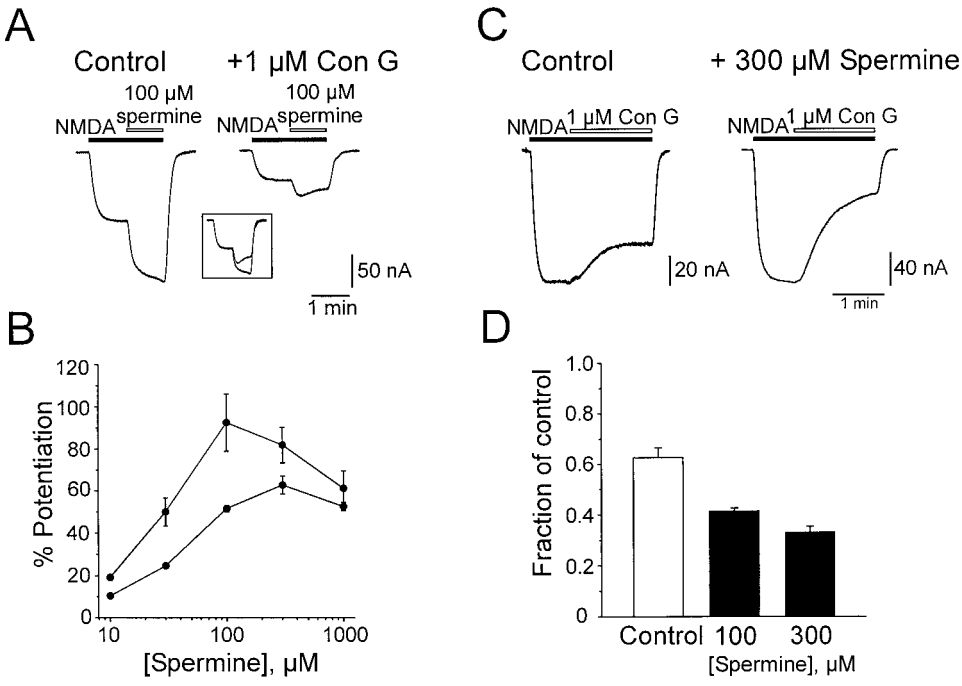

Fig. 9. Polyamines enhance Con G block. Traces in A are recordings from the same oocyte expressing NR1a/NR2B receptors and show spermine potentiation of 100 μM NMDA-evoked (+2 μM glycine) currents in the absence and presence of Con G. The inset in A shows the two traces normalized to the NMDA-evoked current response. Graph in B plots the concentration dependence of spermine potentiation of NMDA-evoked currents from NR1a/NR2B-expressing oocytes in the absence and presence of Con G by using the experimental protocol shown in A (n = 4–6 oocytes at each concentration). Traces in C are from an oocyte expressing NR1a/NR2B receptors and show Con G block of 100 μM NMDA (+2 μM glycine) currents in the absence and presence of spermine. Graph in D plots the steady-state block of the NMDA current by Con G in the absence and presence of increasing concentration of spermine by using the experimental protocol shown in C (n = 4–6 oocytes/treatment group).

Although Con G did produce an apparent noncompetitive-like shift in the concentration-response relationship for spermine potentiation of NMDA responses, this is likely the result of a spermine enhancement of Con G block as opposed to Con G modulation of spermine potentiation. In support of this possibility, Con G block was enhanced in the presence of spermine, and a similar spermine-induced enhancement of block was seen with the competitive antagonist APV. Finally, previous electrophysiological and binding studies have shown that spermine will modulate ligand binding to the NMDA recognition site (Reynolds, 1994; Williams, 1994).

Why then is Con G more effective at blocking spermine-enhanced MK-801 binding than glutamate-evoked MK-801 binding? The answer may lie in the selectivity of Con G for receptors containing the NR2B subunit. Developmental and pharmacological studies have demonstrated that in forebrain structures the NR2 subunit expression changes during development. Although the NR2B subunit expression predominates at fetal and neonatal time periods, there is a gradual increase in expression of the NR2A subunit with age. Thus, it is likely that in the adult NMDA receptors contain both NR2A and NR2B subunits, and that NMDA receptors containing only the NR2B (with the NR1 subunit) make up a smaller fraction of the total population of NMDA receptors compared with young animals. This is supported by recent immunoprecipitation studies where it has been reported that in the adult rat the dominant fraction of NMDA receptors contains both NR2A and NR2B subunits. Much smaller fractions contain the individual NR2 subunits (Luo et al., 1997; but see Blahos and Wenthold, 1996; Chazot and Stephenson, 1997). In addition, in electrophysiological studies the NR2B-selective antagonists ifenprodil and CP101,606 produce potent block of NMDA responses in fetal and neonatal tissues and have little or much less of an effect in the adult (Kirson and Yaari, 1996; Stocca and Vicini, 1998), which suggests that the relative proportion of NMDA receptors containing just the NR2B subunit is very low in the adult rat. The [^{3}H]MK-801 binding studies with Con G were carried out with adult brain tissue. If Con G is specific for NR2B-containing receptors and if the expression of NMDA receptors containing just the NR2B subunit is relatively low in the adult tissue, one would not expect Con G or other NR2B-selective ligands to have much effect on basal or NMDA-stimulated binding. However, spermine would selectively potentiate MK-801 binding to NR2B-containing receptors, thus amplifying NR2B-dependent responses. Under these conditions Con G should produce very potent blocking effects as was observed. The enhancement of Con G block by polyamines may have functional implications. Polyamines are released in a calcium-dependent manner from neurons in response to chemical, electrical, or K^+ stimulation (Harman and Shaw, 1981; Gilad and Gilad, 1991; Fage et al., 1992). Moreover, seizures and ischemia have been associated with an increase in polyamine levels (Hayashi et al., 1993; Carter et al., 1995). The activity-dependent release of polyamines may serve to enhance Con G block during periods of intense neuronal activity such as that occurring during seizures or stroke.

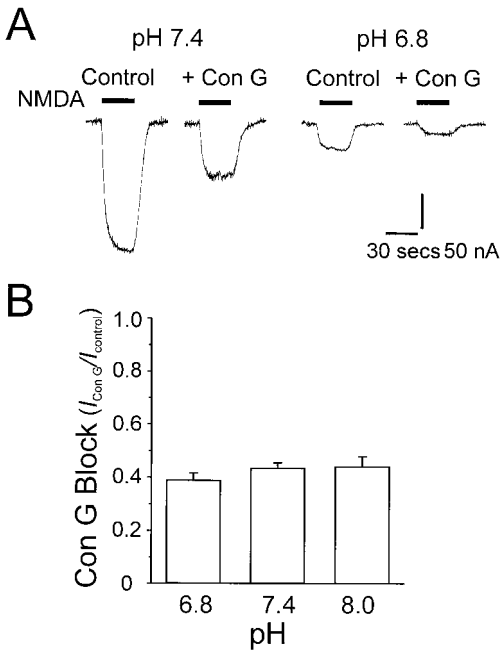

Fig. 10. Protons do not affect Con G block. Traces in A are from an oocyte expressing NR1a/NR2B receptors and show 100 μM NMDA (+2 μM glycine) before and after steady-state block by Con G at normal (7.4, left traces) and low (6.8, right traces) pH. Data similar to and including those shown in A are summarized in B (n = 4–6 oocytes tested at each pH).

This is the first truly selective NR2B subtype-specific competitive antagonist at the NMDA-binding site. Earlier studies demonstrated that competitive antagonists were relatively weak at NR2C- and NR2D-containing receptors, and showed minimal selectivity for NR2B- versus NR2A-containing receptors, being slightly more potent at NR2A than NR2B (Buller et al., 1994; Buller and Monaghan, 1997). However, we have recently demonstrated that a related conantokin isolated from *Conus radiatus* (Con R) will block receptors containing NR2A or NR2B, and has less of an effect at receptors containing the NR2C or NR2D subunits (White et al., 2000). Finally, a conantokin isolated from another species of cone snail will block receptors that contain the NR2D subunit in addition to blocking receptors that contain either the NR2A or NR2B subunit (S. D. Donevan, unpublished observations). This raises the possibility that by using this peptide backbone one may be able to develop a panel of subunit-specific antagonists selective for each of the NR2 subunits. These compounds would be useful both from the standpoint as tools for examining NMDA receptor function and as therapeutic agents targeted toward neurological disease. The NR2B subunit-selective action of Con G, in particular, may underlie its favorable side effect profile of Con G compared with other competitive antagonists in behavioral studies. For instance, Con G has been shown to have potent anticonvulsant activity after i.c.v. administration in a range of seizure models (Armstrong et al., 1999). Moreover, there was a large separation between protective doses and those which produce toxicity [protective index of (TD_{50}/ED_{50}) ~27 in the Frings audiogenic seizure-susceptible mouse model] compared with other NMDA receptor antagonists and standard anticonvulsants. Although its limited bioavailability prevents systemic administration of Con G for treatment of neurological disorders (S. White, unpublished observations), it may be possible to bypass the blood-brain barrier and deliver Con G directly into the brain. A similar approach is being taken with the conus peptide SNX 111 for treatment of neuropathic pain.

Acknowledgments

We thank Drs. Heinemann and Seeburg for providing the NMDA receptor clones, Cognetix, Inc. for providing Con G, David Adams and Greg Campbell for technical assistance, and Dr. Baldomero Olivera for helpful discussions.

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