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Voltage-Dependent Block by Strychnine of *N*-Methyl-D-Aspartic Acid-Activated Cationic Channels in Rat Cortical Neurons in Culture

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SUMMARY

Single-channel currents were recorded by means of the patch clamp method in outside-out patches excised from rat cortical neurons in primary culture. The excitatory amino acid *N*-methyl-D-aspartic acid activated mainly 40–50 pS conductance channels. Channel opening durations were characterized by a series of rapid openings and closures induced by the presence of Mg^{2+} ions. This inhibitory effect was voltage dependent. Strychnine, the antagonist of the glycine-gated Cl^- channels, blocks the *N*-methyl-D-aspartic acid-activated cationic channel in cultured rat cortical neurons. Strychnine action is voltage dependent and it is not counteracted by ethylenediaminetetraacetic acid, ruling out Mg^{2+} contamination of strychnine. It has been reported recently that glycine increases the rate of openings of *N*-methyl-D-aspartic acid-activated channels. This action is not affected by the presence of strychnine. Our results show that 1) Mg^{2+} and strychnine have an apparently similar intermediate blocking action on the NMDA-activated channels, 2) strychnine presumably acts as a sequential open channel blocker producing a different type of block compared with the one reported for Mg^{2+} ions, and 3) the lack of effect of strychnine on the glycine potentiation of the *N*-methyl-D-aspartic acid response indicates that this alkaloid does not competitively antagonize glycine but acts as an open channel blocker.

Glycine is one of the major inhibitory neurotransmitters in the spinal cord and brain stem of vertebrates and many invertebrates (1, 2). Strychnine binds with high affinity to synaptic membranes of rat brainstem and spinal cord (3), which are closely related to the distribution of strychnine-displaceable, high affinity, glycine binding sites (4). Moreover, strychnine selectively antagonizes the inhibitory action of glycine on spinal cord and brainstem neurons (5). These findings have suggested that strychnine binding represents binding to the glycine receptor, and strychnine has been used in studies of interactions with this receptor (6) and in its purification and biochemical characterization (7).

Strychnine exerts multiple actions not associated with a glycinergic site (see Refs. 8 and 9 for review), although questions about specificity arise mostly because of the high doses used. It is also not clear whether the component of total strychnine binding not displaceable by glycine has pharmacological significance (6, 10). In spite of these results, the possibility of a secondary action of strychnine cannot be ruled out.

Recently, Johnson and Ascher (11) have demonstrated that glycine potentiates the NMDA-activated channel current in cortical neurons and that this potentiation is not mediated by the inhibitory strychnine-sensitive glycine receptor. This effect is strychnine resistent and consists of an increase in the rate of NMDA-activated channel openings.

NMDA is an agonist of the glutamate receptor (12, 13) and in cortical neurons, at 2 μM concentration, activates channels of about 40–50 pS conductance, mean open time 6 msec, and rate of channel opening 1 sec^{-1} (14). In Mg^{2+}-free solution, the current to voltage relation is linear and the reversal potential is near 0 mV, whereas the presence of Mg^{2+} produces a decrease of current at negative membrane potentials, yielding a negative-slope current to voltage relation in the region of these potentials. This indicates a voltage dependence of the Mg^{2+} block (15).

In the present paper, we report an interaction between strychnine and the NMDA-activated cationic channel. Strychnine decreases the mean channel open time and the rate of channel opening. The observed channel block is apparently similar to the one elicited by Mg^{2+}, i.e., fast flickering of the channel between the open and closed states. A decrease in the rate of NMDA-activated channel openings by Mg^{2+} ions has been reported (15). In contrast, strychnine fails to affect the glycine-induced channel opening frequency increase. The ac-

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

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tion of strychnine in comparison with Mg^{2+} on NMDA-activated channels will be discussed.

Materials and Methods

Cell cultures. Primary cultures of cortical neurons from newborn rats were prepared with a modification of the dissociation procedure described by Levi *et al.* (16) for postnatal rat cerebellar neuronal cultures. Briefly, cortical cells from newborn rats were dissociated with trypsin (Sigma Chemical Co., St. Louis, MO), 0.25 mg/ml, and plated at a density of 800,000 cells/dish on 35-mm Nunc dishes coated with 10 μg/ml poly-L-lysine (Sigma). Cells were grown in basal Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO), 25 mM KCl, 2 mM glutamine (Sigma), and 100 μg/ml gentamicin (GIBCO) for 1 to 3 weeks. Cytosine arabinofuranoside (1 μM; Sigma) was added 24 hr after plating to inhibit glial cell replication.

Electrophysiology. Single NMDA-activated channel currents were recorded in the outside-out configuration by means of the patch clamp method (17). The experiments were performed at room temperature (21–23°) using a recording medium composed of (mM) NaCl, 145; $CaCl_2$, 1; glucose, 5; HEPES-NaOH, 5; at pH 7.4. Continuous perfusion (1 ml/min) of the dish by gravity was performed to avoid drug accumulation. The pipette solution contained (mM) $CsSO_4$, 145; $CaCl_2$, 1; EGTA, 1.1; HEPES-CsOH, 10; at pH 7.2. Blunt pipettes were used to apply the drugs by leakage when positioned close to the outside-out membrane patch. In parallel whole-cell voltage-clamp experiments, NMDA, kainic acid, and quisqualic acid (Cambridge Research Biochemical, Cambridge, England) were pressure applied (1–3 psi) onto the soma of cortical neurons. Strychnine, dissolved in the recording solution, was perfused at a high rate (10 ml/min).

Data analysis. Records of single-channel currents were sampled at 10 kHz sampling rate using the Indec data acquisition system (Sunnyvale, CA) after filtering with a low-pass, eight-pole Bessel filter set at 3 kHz and stored in the memory of a Digital Equipment Corporation LSI 11/73 computer. A semiautomated procedure (18) generated distributions of open times. The threshold setting to validate channel opening and closing was determined by using half the amplitude of the channel current. Single exponential fitting of the distribution of kinetic parameters with probability density curves was performed using the method of maximum likelihood, optimization of fit being provided by a Simplex algorithm.

In noise analysis experiments, power spectra were obtained from single-channel records at a sampling frequency of 2 kHz after filtering with a low-pass Butterworth filter (Frequency Devices, MA) with corner frequency at 0.9 kHz. An iterative, nonlinear least squares regression routine fitted the experimental spectra with single or double lorentzian curves. The time constants (τ values) were derived from each corner frequency. More detailed description of the methods used for noise analysis can be found elsewhere (19).

Results

Strychnine acts on the NMDA-activated channel. In outside-out patches excised from neonatal rat cortical neurons, at a holding potential of −50 mV and in a Mg^{2+}-free solution, NMDA (10 μM) activates a homogeneous population of channels with a mean open time of 5–6 msec and a conductance of about 50 pS. In the presence of strychnine (20 μM), flickering between the open and closed states was observed (Fig. 1B), indicating a blocking action on the NMDA-activated channel. As illustrated in Fig. 1, strychnine acts as a voltage-dependent blocking agent. When the holding potential of the patch is inverted at positive values, the single channel currents are identical to those recorded in the absence of Mg^{2+} or strychnine.

In the presence of Mg^{2+}, channel openings exhibit the same amplitude but with a flickering behavior, indicating blocking action of the ion (15). Single-channel inward currents induced by 10 μM NMDA in the presence of Mg^{2+} (20 μM) are shown in Fig. 2A. EDTA (5 mM), a chelating agent for divalent cations, counteracts the action of Mg^{2+}, preventing channel block (Fig. 2B). In the presence of EDTA we observed an increase of channel conductance. Spontaneous channel openings in the absence of NMDA were observed when EDTA was present. These openings were abolished (data not shown) by amino-phosphono-valeric acid (100 μM) and phencyclidine (50 μM), both NMDA antagonists. Possibly these openings were due to contamination with endogenous glutamate and/or glycine or they could be due to an agonist action of EDTA itself. We did not investigate this interaction further. The channel openings elicited by simultaneous application of strychnine (20 μM), EDTA (5 mM), and 10 μM NMDA show the same flickering

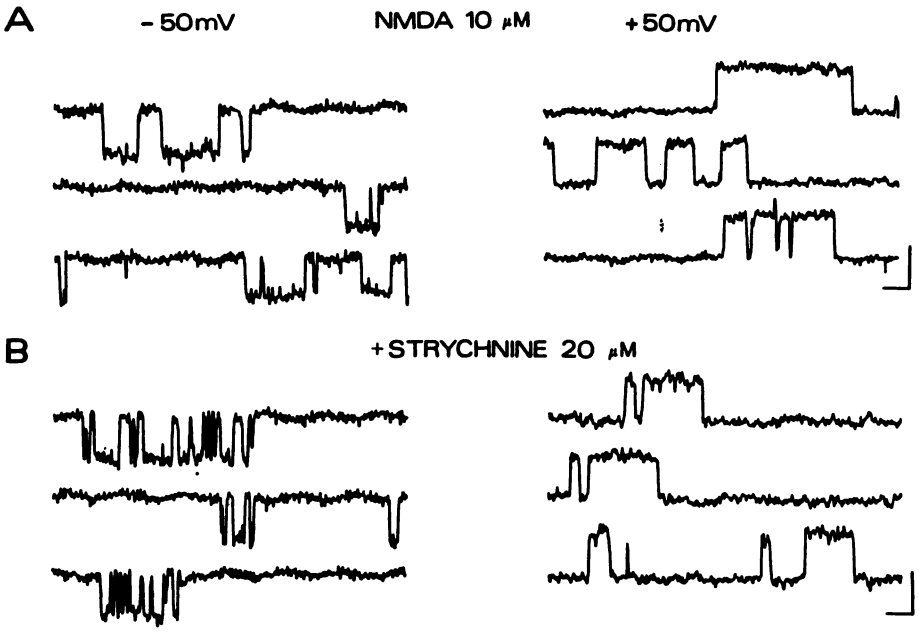

Fig. 1. NMDA (10 μM)-activated channel openings, in the absence of (A) and in combination with (B) strychnine (20 μM) at a negative and a positive patch holding potential of ±50 mV show lack of blocking action at a positive holding potential. *Bars*, 2.5 pA and 2 msec.

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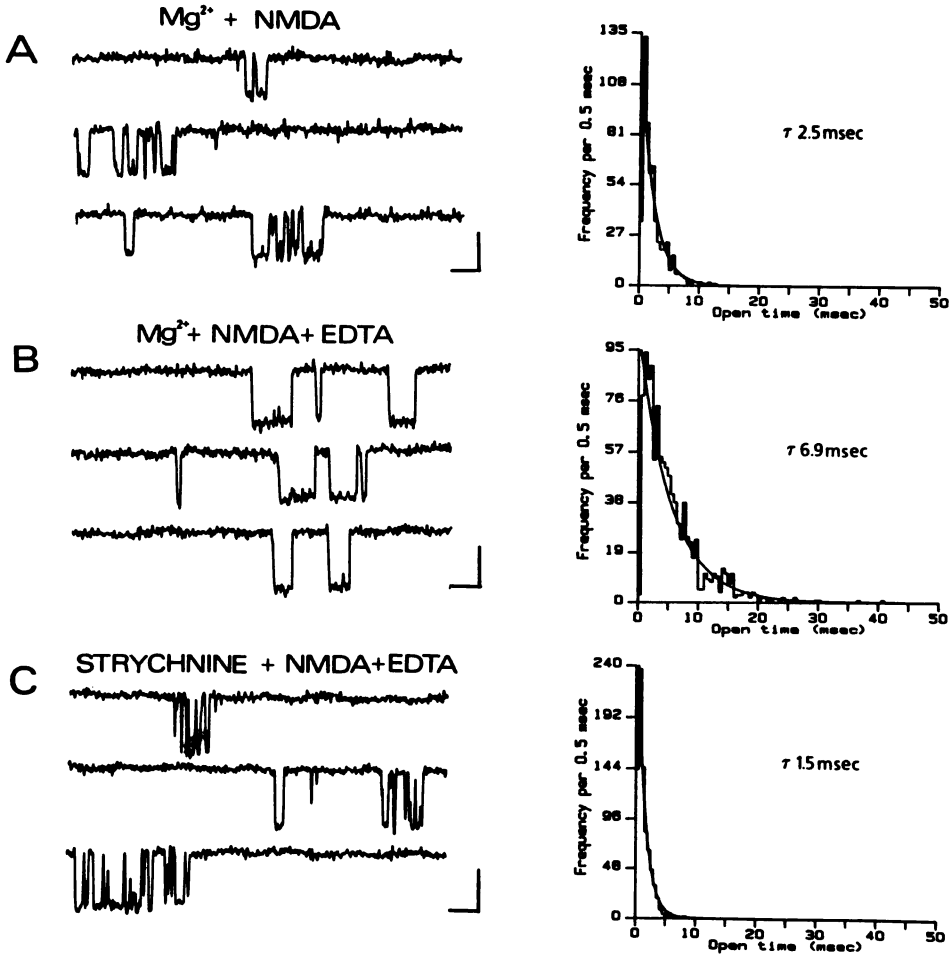

Fig. 2. Cationic channels activated by 10 μM NMDA at a holding potential of −50 mV in an outside-out patch excised from a rat cortical neuron. To the *right* of each recording is shown the channel open time distribution for that record. A, The activity of NMDA is counteracted by the presence of 20 μM Mg^{2+}. B, The combined action of Mg and EDTA (5 mM) fails to block the NMDA-activated channel. C, Strychnine (20 μM) in the presence of 5 mM EDTA blocks the channel openings by inducing a flickering between the open and closed states. Note the increase of channel amplitude in presence of EDTA (see text). Calibration *bars* are 2.5 pA and 5 msec.

behavior (Fig. 2C), indicating a strychnine action apparently similar to but not mediated by Mg^{2+}, leading to the reduction of channel opening duration. This is shown in Fig. 2, in which the open time distribution histograms are derived from openings recorded in the three conditions. On average the mean channel open time was 6.1 ± 1 msec with 10 μM NMDA alone, 2.5 ± 0.9 msec when 20 μM Mg^{2+} was added, and 1.8 ± 0.9 in the presence of 20 μM strychnine (mean ± SD, n = 6 patches).

Power spectra (Fig. 3) of NMDA-induced channel currents in an outside-out patch held at −50 mV, obtained during the simultaneous application of Mg^{2+} and EDTA, are well fitted by a single lorentzian curve (Fig. 3A) yielding a time constant of 7 ± 2 msec (mean ± SD, n = 4 patches). In the presence of strychnine and EDTA the power spectrum is, instead, best fitted by the sum of two lorentzians (Fig. 3C). The two time constants are 18 ± 4 msec and 0.4 ± 0.3 msec (mean ± SD, n = 6 patches). In the presence of Mg^{2+} and the absence of EDTA, the power spectrum was also fitted by two lorentzians (Fig. 3B) a fast one, 0.5 ± 0.2 msec, and a slower one, 6 ± 2 msec (mean ± SD, n = 6 patches). The time constants of the slower components of the power spectra in the presence of Mg^{2+} or in the presence of strychnine were significantly different ($p < 0.05$, paired t test).

Specificity of the strychnine action on the NMDA-induced response. Strychnine (20 μM) also produces a reduction (Fig. 4) of the rate of channel openings activated by NMDA in an outside-out patch held at −50 mV and a decrease of the total current elicited by NMDA (Fig. 5A) in rat cortical neurons that are voltage clamped using the whole cell configuration. The decrease of channel opening rate was 33 ± 16% (decrease from the control value of 2.6 ± 0.5 openings/sec, mean ± SD, n = 6 patches, 10 μM NMDA, 20 μM strychnine). The amplitude of whole cell NMDA-induced current was reduced on the average by 27 ± 6% (n = 5 cells). It is clear that the reduction of the mean channel open time and channel opening frequency are the mechanisms responsible for the decrease of the total current. Pressure application of NMDA, kainic acid, and quisqualic acid, three glutamate receptor agonists (20), produces inward currents in voltage-clamped cortical neurons. Rapid perfusion of the recording dish with strychnine (30 μM) selectively reduced only the NMDA response (Fig. 5), in analogy with what was observed with Mg^{2+} ion applications (15). Kainate and quisqulate responses were unaffected, being 103 ± 10% and 97 ± 15% of control, respectively (n = cells). A higher concentration of strychnine (60 μM) decreased only the NMDA response and failed to affect the kainic and quisqualic acid responses (data not shown).

Interaction of glycine with strychnine. Glycine exerts a positive allosteric modulatory action on glutamate receptors (NMDA subtype) by increasing the NMDA-activated channel opening frequency in outside-out patches obtained from fetal mouse cortical neurons in culture (11). We investigated the combined action of glycine and strychnine on the NMDA-activated channels. In six experiments, the positive modulatory

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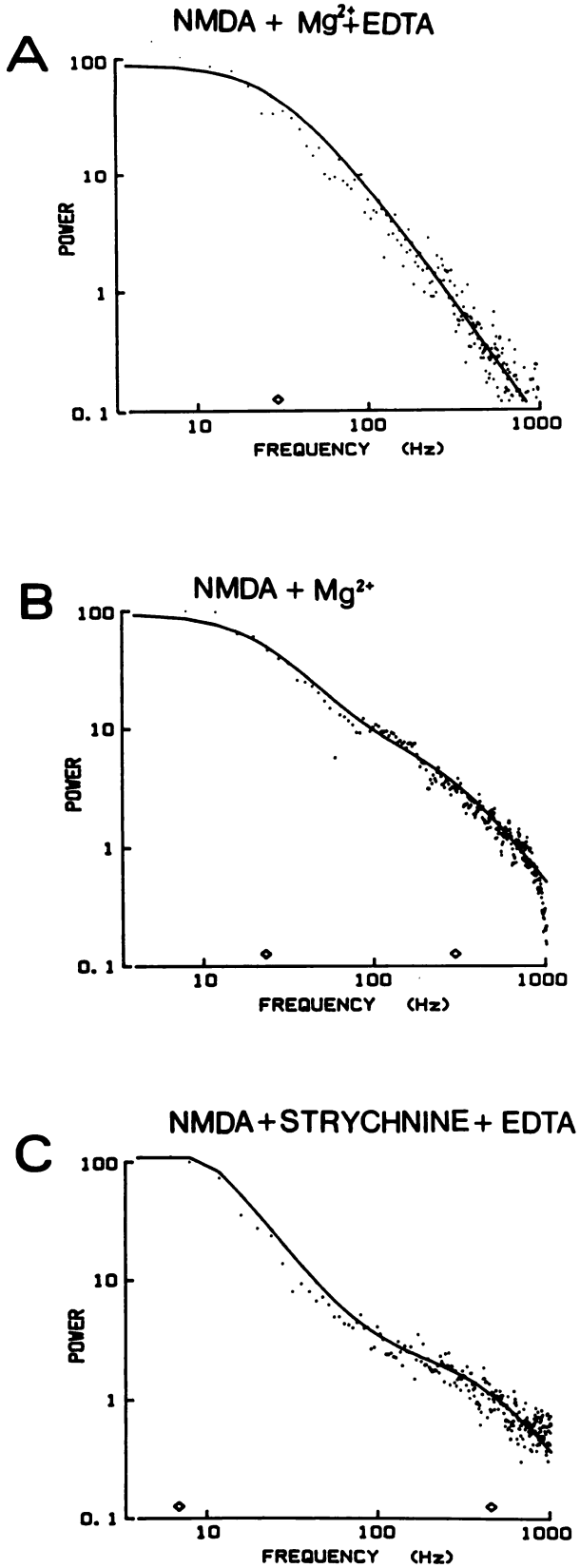

Fig. 3. Noise analysis of 10 μM NMDA-activated channel currents from an outside-out patch held at −50 mV. A, Power spectrum obtained in presence of 20 μM Mg^{2+} and 5 mM EDTA, best fitted by a single lorentzian curve yielding a time constant of 6 msec. B, Power spectrum obtained in the presence of 20 μM Mg^{2+} best fitted by the sum of two lorentzians with time constants of 0.4 and 6.2 msec. C, Power spectrum obtained in the presence of 20 μM strychnine and 5 mM EDTA, best fitted by the sum of two lorentzians with time constants of 0.3 and 20 msec.

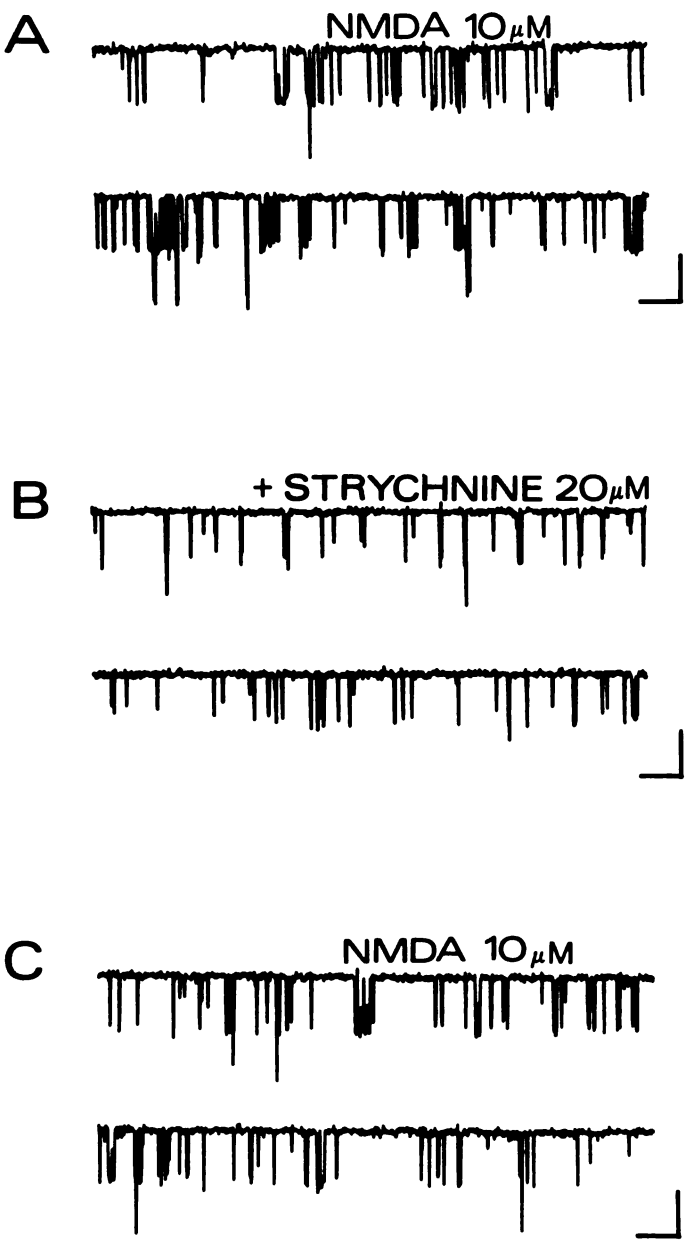

Fig. 4. Channel activity evoked by 10 μM NMDA displayed using a slow time scale to show variations of the rate of channel opening in the absence of (A) or in combination with (B) 20 μM strychnine and (C) recovery. *Bars*, 2.5 pA and 1 sec.

action of glycine (1 μM) was not affected by the presence of strychnine (20 μM) (data not shown), as has already been reported for lower (1–10 μM) strychnine concentrations (11). We did not investigate the decrease produced by strychnine on the combined application of glycine and NMDA.

Discussion

We investigated the interaction of strychnine with the NMDA receptor in outside-out patches excised from cortical neurons. High affinity binding and antagonistic action of strychnine on a glycine recognition site coupled to an anion-selective channel is well known (3, 5, 6). However, in regions of the central nervous system different from spinal cord and brainstem, glycine-resistant strychnine binding and activity

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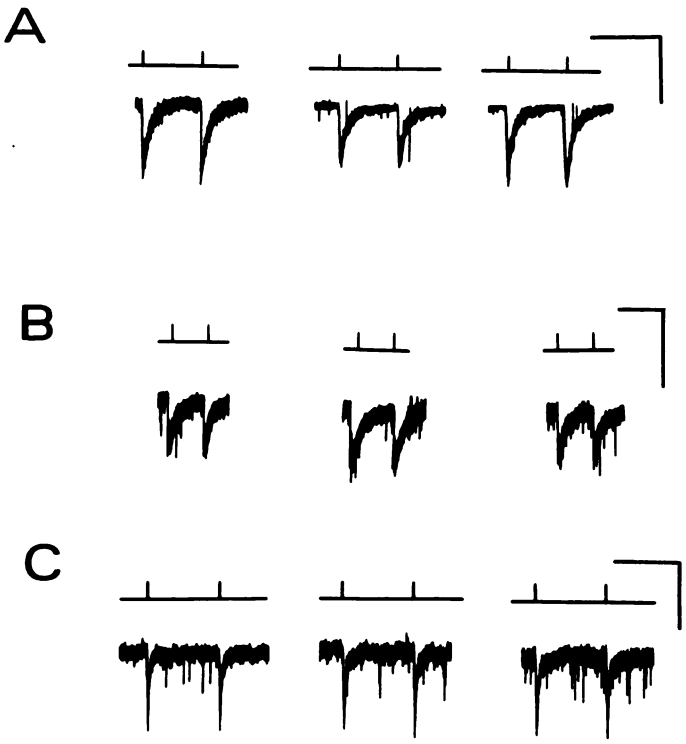

Fig. 5. NMDA (A), quisqualate (B), and kainate (C) induced inward currents in a voltage-clamped cortical neuron at −50 mV. All agonists had a concentration of 50 μM. The addition of strychnine (30 μM) did not affect the currents induced by quisqualate and kainate, whereas it decreased the currents evoked by NMDA. Calibration *bars* are 50 pA and 5 sec.

have been reported (8, 9, 10). It is not clear whether there is any pharmacological significance of the glycine-resistant action of strychnine. The discovery of a second type of glycine receptor in the central nervous system (11) as a regulatory site for the NMDA-activated channel has prompted the question of strychnine sensitivity for this glycine action. It is clear that strychnine fails to interfere, at least at the tested concentration, with glycine potentiation of the NMDA response.

We confirm these results and we show evidence that strychnine is acting on the NMDA-activated channel probably as an open channel blocker. Strychnine produces both fast openings and closures (flickering) of the open channel and a reduction in the rate of channel openings similar to those obtained with Mg^{2+} ions. A major difference, however, was noted from noise analysis of single NMDA-activated channel currents in the presence of strychnine and Mg^{2+}. The slow time constant of power spectra derived from single-channel currents reflects the burst duration (21) of the NMDA-activated channel. Mg^{2+} block produced an additional fast component in the power spectrum, leaving the slower one unaffected. It has been shown (15) that the Mg^{2+} blockade does not produce an increase in channel burst duration. Strychnine block, instead, increased the time constant of the slow component of power spectra in a significant manner. The increased burst duration induced by strychnine leads to the possibility of a sequential type of channel block, similar to that described (22) for local anaesthetic action at the nicotinic cholinergic channel. This was shown clearly not to be the case for Mg^{2+} ions (15). Furthermore, the possibility of Mg^{2+} contamination of the strychnine solution was ruled out by experiments using EDTA to chelate Mg^{2+} and other divalent cations. Even under these experimental conditions strychnine was active in blocking the channel. Like that of Mg^{2+}, this action is voltage dependent and highly specific for the NMDA subtype of glutamate receptor, not for the quisqualate and kainate subtypes. Kainate and quisqualate, which preferentially open a smaller conductance cationic channel (12, 13), appear to be strychnine insensitive. The concentrations of strychnine needed to produce a pronounced reduction of the NMDA response are necessarily very high, at least 100-fold the concentration that blocks the strychnine-sensitive glycine receptor. This is in good agreement with the existence of only one high affinity strychnine binding site (3, 6), the one coupled to the glycine receptor-Cl^- channel. Whether there is any pharmacological relevance of these actions of strychnine is dubious, especially because in the cerebrospinal fluid, Mg^{2+} ions are already present at millimolar concentrations. In any case, this regulatory site for the NMDA subtype of glutamate receptors can also be the target of other drugs, possibly those with higher affinities or endogenous modulators.

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