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ACCELERATED COMMUNICATION

Chloride Transport Blockers Prevent *N*-Methyl-D-aspartate Receptor-Channel Complex Activation

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SUMMARY

In cultured spinal cord neurons, we found that blockers of chloride transport (furosemide, a widely used loop diuretic, and the related compounds piretanide and bumetanide, as well as niflumic and flufenamic acids, used as antiinflamatory agents) prevented *N*-methyl-D-aspartate (NMDA) receptor activation in a dose-dependent manner and are specific for this class of glutamate receptor. Antagonism of NMDA-mediated currents by chloride transport blockers was voltage independent and showed fast on-off kinetics. The action was noncompetitive with NMDA and did not arise from interaction with the Zn^{2+} inhibitory site, because blockade of NMDA-induced responses by furosemide and Zn^{2+} was additive. The inhibition was greater in a low concentration of glycine, but it could not be overcome by increasing the glycine concentration (up to 100 μM). In contrast, the inhibition was attenuated by the polyamine spermine. Because the presence of spermine was not required for inhibition to develop, we conclude that chloride transport blockers are noncompetitive antagonists of the NMDA receptor, likely acting as inverse agonists of the polyamine site. This action may explain the protective effect that has been shown for some of these drugs in neuronal degeneration; because they also prevent neuronal swelling, they may be good starting compounds for synthesis of appropriate therapeutic agents to ameliorate excitotoxicity.

Among the postsynaptic receptors activated by the excitatory amino acid glutamate, the NMDA-preferring receptor is involved in complex physiological processes such as long term potentiation (see Ref. 1 for review), learning (2), and development structuring (3, 4). This receptor is also involved, through an excitotoxic process, in central nervous system pathology, including ischemic cell death, epilepsy, and other neurological diseases (5, 6). For this reason, any compound having agonist or antagonist activity at the NMDA receptor becomes a potential therapeutic agent. The NMDA receptor has, in addition to the agonist binding site, at least four distinct binding domains at which interference can modify receptor activity, 1) the glycine binding site, 2) the Zn^{2+} binding site, 3) the ion channel itself, at which Mg^{2+} and phencyclidine produce voltage-dependent and use-dependent blockade (7, 8), and 4) the recently discovered polyamine binding site (9). These regulatory sites allow for the possibility of developing potential clinically useful compounds for preventing glutamate-mediated injury. Protection from excitotoxic damage has been effectively demonstrated for a number of NMDA receptor antagonists (10).

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We have found that inhibitors of chloride transport, widely used clinically as diuretics and anti-inflammatory agents, are able to antagonize NMDA-induced currents while minimally affecting activity at other glutamate receptor subtypes, the kainate and the AMPA receptors. The combined action of these compounds on Cl^- fluxes and NMDA receptor activity may have profound implications in preventing excitotoxicity-induced neurodegeneration. Some of these results have been published in abstract form (11).

Experimental Procedures

Cell culture. Spinal cords were dissected from E14 rat embryos and mechanically dissociated after trypsin treatment (0.02 mg/ml, 10 min, 37°). Cells were seeded at a density of 200–300 cells/mm^2, on glass coverslips or in 35-mm Petri dishes that had been previously treated with poly-lysine (10 mg/ml) for 1 hr and incubated overnight with schwanoma-conditioned medium (4°). The cultures were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and antibiotics, in a humidified incubator at 37° and 5% CO_2.

Recording and perfusion techniques. Currents activated by excitatory amino acids were measured in the whole-cell configuration of the patch-clamp technique (12) in well isolated cells (7–20 days in

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ-aminobutyric acid.

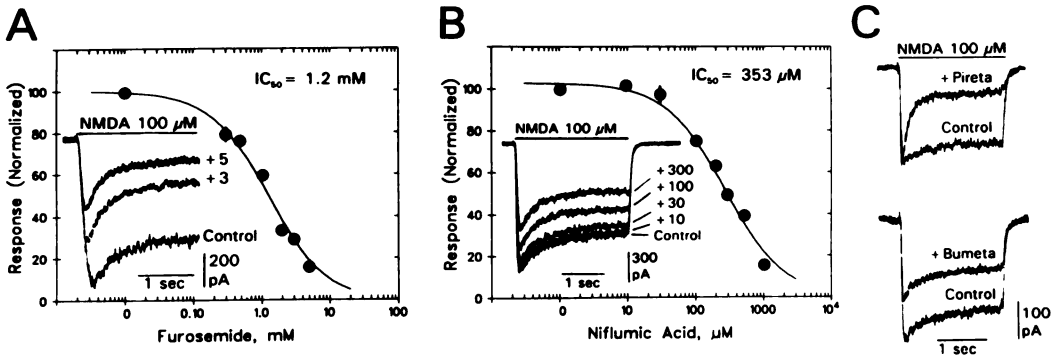

Fig. 1. Inhibitors of chloride transport blocked the current induced by perfusion of NMDA. Complete dose-inhibition curves were constructed for furosemide (A) and niflumic acid (B) and fitted to the equation $I/I_{control} = (1 + [Ant]/IC_{50})^{-1}$, where I is current, $[Ant]$ is the concentration of antagonist, IC_{50} is the half-inhibitory concentration. *Points* represent the mean ± standard error of five and six neurons in A and B, respectively. *Insets*, superimposed averaged (n = 3) records. C, inhibition of NMDA-induced currents by coapplication of piretanide (*Pireta*) (5 mM) and bumetanide (*Bumeta*) (2 mM). In all cases, 10 μM glycine was present and the membrane potential was clamped at −60 mV.

culture), using a L/M-EPC 7 amplifier. Responses to glutamate receptor agonists were filtered (1–3 kHz), acquired (512-Hz sampling rate), and averaged (n = 3–5) on-line, using a personal computer. The computer also controlled a motorized device to move rapidly an array of six flow pipettes situated 100–200 μm from the cell soma. Only two pipettes were flowing at the same time. Experiments were performed at room temperature (20–22°).

Experimental solutions. The external solution was (in mM) 165 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.5), to which strychnine (1 μM) and glycine (at indicated concentrations) were added. Mg^{2+} was omitted to prevent block of NMDA receptor channels. Pipettes contained (in mM) 130 CsCl, 20 CsOH, 10 EGTA, 0.5 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES, buffered to pH 7.2. In some experiments, K^+ substituted for Cs^+ and Cl^- was replaced by methanesulfonate. No marked run-down of NMDA currents was seen during the recording period (up to 20 min); therefore, an ATP-regenerating solution was not included. NMDA, kainate, glycine, strychnine, furosemide, niflumic acid, flufenamic acid, and spermine were purchased from Sigma (St. Louis, MO). AMPA was purchased from Tocris Neuramin (Essex, UK). Piretanide and a sample of furosemide were kindly provided by Höechst Ibérica. Bumetanide was a gift of Boehringer Ingelheim (Spain).

Results

Blockers of chloride transport inhibited both the peak of NMDA-induced response and the smaller steady state current reached after receptor desensitization. None of these compounds, by themselves, had any action on resting currents, at the highest concentration used (data not shown). Fig. 1 shows the inhibitory action on responses elicited by rapid perfusion of NMDA plus glycine. NMDA-induced currents, measured at steady state, were reduced in a dose-dependent manner by furosemide, with an IC_{50} of 1.23 ± 0.06 mM (mean ± standard error, n = 5) (Fig. 1A). Both niflumic and flufenamic acids depressed the NMDA-induced currents more potently. IC_{50} for niflumic acid was calculated to be 353 ± 68 μM (n = 6) (Fig. 1B). Complete dose-inhibition curves were not obtained for flufenamic acid, but at 200 μM it was equipotent with niflumic acid (31 ± 6% and 35.1 ± 3% of inhibition for flufenamic and niflumic acids, respectively; n = 4). Like furosemide, the structurally related compounds piretanide and bumetanide also inhibited the NMDA-induced response, although with slightly less potency (Fig. 1C). Piretanide, at the highest concentration tested (5 mM), depressed the steady state response to NMDA by 70% (84% at 5 mM furosemide), and 2 mM bumetanide produced an inhibition of 54% (67.6% at 2 mM furosemide). Currents induced by perfusion of kainate and AMPA were

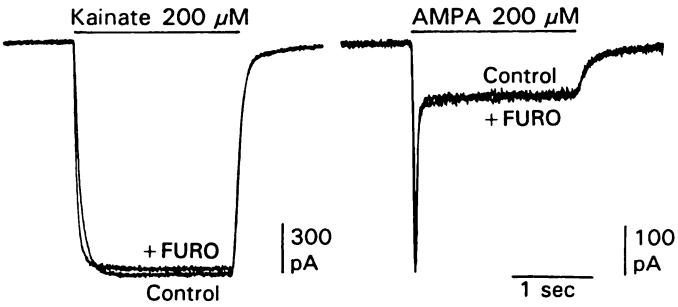

Fig. 2. Lack of effect of furosemide (*Furo*) on non-NMDA glutamate receptors. Currents induced by application of kainate or AMPA were minimally modified by inclusion of 5 mM furosemide. Superimposed responses are the average of three trials in each case.

modified only slightly by chloride transport inhibitors (Fig. 2). Kainate-induced current in the presence of 5 mM furosemide was 91.5 ± 1% of control (n = 14). AMPA-induced responses were characterized by a fast current relaxation that rapidly decayed to a markedly smaller steady state level. In the presence of 5 mM furosemide the average steady state response to AMPA was 110 ± 8% of control (n = 10).

The inhibition elicited by the chloride transport blockers developed in few milliseconds. An example of the rate of inhibition onset and offset is shown in Fig. 3. In neurons exposed to NMDA and furosemide in the presence of glycine, the removal of furosemide from the perfusion solution resulted in an increase in current to a level that coincided with the amplitude of the steady state current recorded in control responses. The steady state response was again attenuated upon furosemide reapplication. The rapid change of the solution around the cell was critical for determination of the rate of inhibition. In optimal perfusion conditions, current relaxations were well fitted by single exponentials with time constants of 20–30 msec for the onset of inhibition. Recovery was slightly slower (τ = 35–60 msec). Although a detailed kinetic analysis was not performed, these results indicate direct binding to the receptor complex as the mechanism of action for furosemide, because the inhibited state and the recovery were both reached rapidly.

To determine the site(s) at which the chloride transport blockers are acting, we first measured the inhibition by furosemide of responses evoked by low and high NMDA concentrations. The degree of attenuation by 1 mM furosemide on responses to 30 μM NMDA (plus 10 μM glycine) was 36.2 ± 2.5% (n = 7), whereas it was 33.1 ± 2.9% (n = 6) when the response

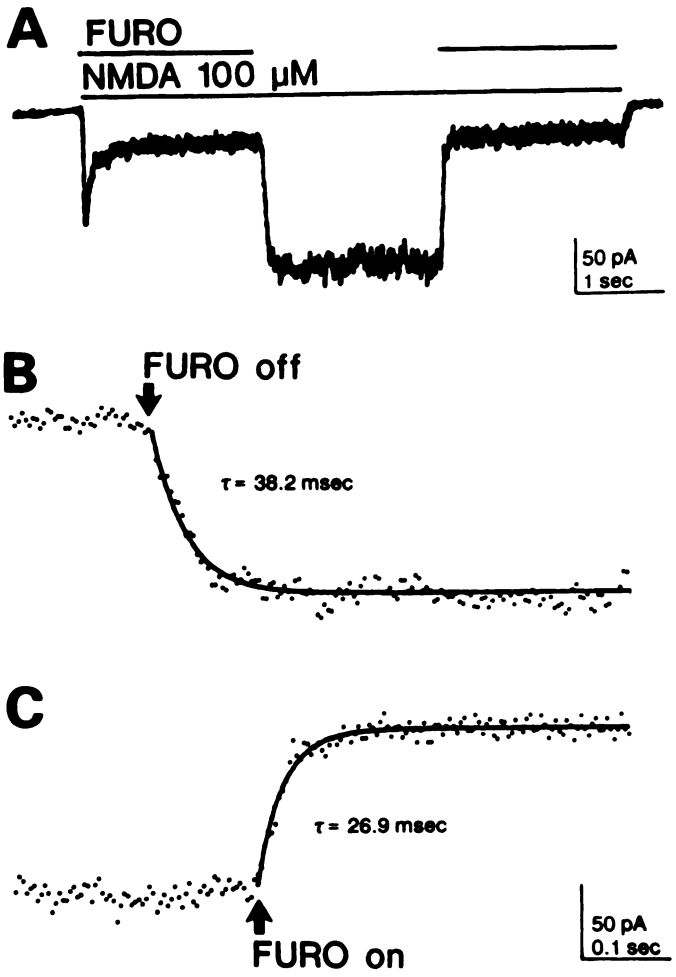

Fig. 3. Kinetics of furosemide (*Furo*) inhibition. A, Furosemide was coapplied with NMDA (plus 10 μM glycine); once steady state block was attained, furosemide was removed from the perfusion fluid for 2 sec and then applied again in the continuous presence of NMDA. The current recovered completely upon furosemide removal. The time courses of recovery (B) and inhibition (C) of NMDA currents are shown on a faster time scale. The offset and onset of inhibition were fast and well fitted by single exponentials (continuous superimposed trace) with the indicated time constants (τ).

was induced by 300 μM NMDA (Fig. 4A). Actually, complete dose-inhibition curves for furosemide were indistinguishable for 30 μM and 300 μM NMDA (IC_{50} = 1.2 ± 0.07 and 1.3 ± 0.1 mM, respectively). In contrast, inhibition of steady state currents was larger with a low than a high glycine concentration. Fig. 4B shows the attenuation by furosemide of NMDA responses induced in the presence of 0.3 μM or 10 μM glycine. Furosemide (0.5 mM) reduced the response to NMDA (100 μM) by 42 ± 1.7% in the presence of 0.3 μM glycine, whereas the inhibition was only 24 ± 1.3% (n = 4) when the glycine concentration was raised to 10 μM. However, the degree of blockade produced by furosemide was unaffected by a further increase in glycine concentration. Responses to NMDA plus 10 or 100 μM glycine were attenuated by 66.7 ± 3.3% and 61.3 ± 2.7% (n = 6), respectively, upon furosemide (2 mM) perfusion. This result clearly indicates that inhibitors of chloride transport are able to modulate the glycine site in a noncompetitive manner.

The antagonism of NMDA-induced currents was not voltage dependent (Fig. 5A), making an interaction of these compounds with the inner channel wall unlikely. Summation experiments involving the allosteric inhibitor Zn^{2+} and furosemide revealed no detectable interaction. Zn^{2+} (20 μM) blocked the response to NMDA by 87 ± 4.4% in the absence and by 88.8 ± 2.1% (n = 4) in the presence of 2 mM furosemide. Fig. 5B shows the inhibitory action of Zn^{2+} with and without furosemide. Both Zn^{2+}-inhibited responses are also shown superimposed after normalization of their respective controls (i.e., responses in the absence of Zn^{2+}) for the steady state current amplitude (Fig. 5, *inset*).

The antagonism by chloride transport inhibitors of NMDA receptors was attenuated by the polyamine spermine. In a series of experiments, NMDA (plus glycine) was perfused in the presence of spermine. The polyamine potentiated the response to NMDA (Fig. 6A) in a dose-dependent manner, with an EC_{50} of 70–80 μM and a Hill coefficient of 1.6–1.7 (with 100 μM NMDA plus 1 μM glycine). In contrast to published results (13–15), we did not find a clear bell-shaped dose-response curve for spermine (up to 0.5 mM) (data not shown). Fig. 6B shows that spermine shifted the dose-inhibition curve for niflumic acid to

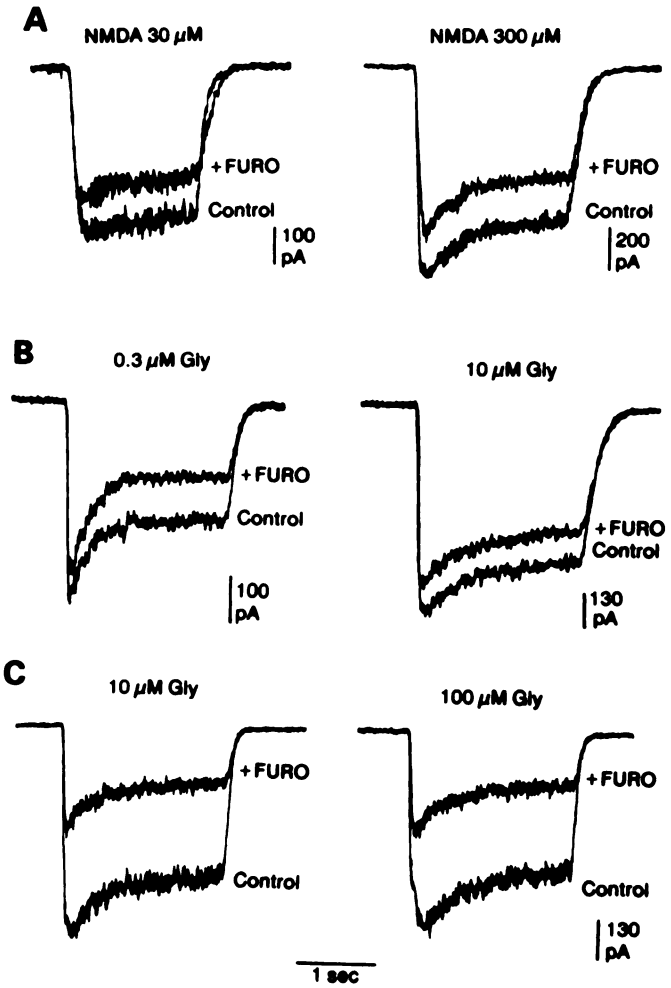

Fig. 4. A, Inhibitory action of 1 mM furosemide (*Furo*) on responses evoked by 30 and 300 μM NMDA (plus 10 μM glycine). The degree of attenuation was similar in both cases. B, The inhibition of response to NMDA (100 μM) by 0.5 mM furosemide was smaller in 10 μM than in 0.3 μM glycine (*Gly*). C, The inhibition of NMDA responses by 2 mM furosemide could not be surmounted by increasing the glycine concentration up to 100 μM. Responses are averages of three trials. B and C are from different cells.

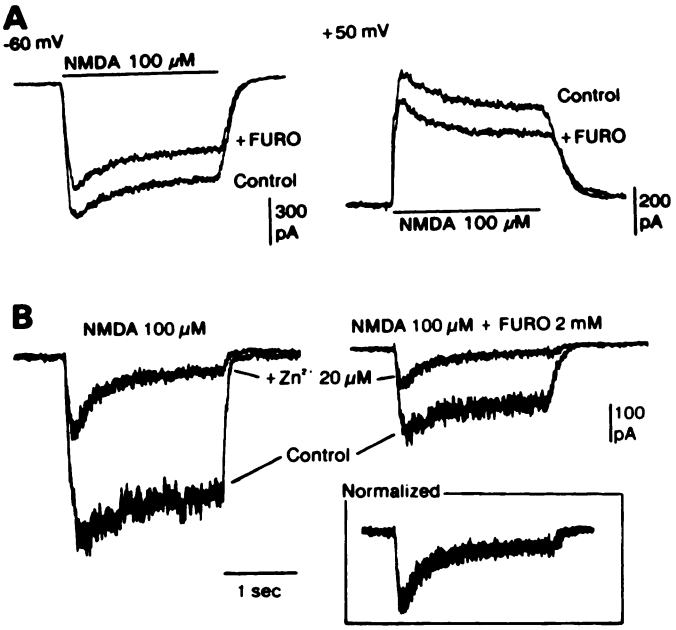

Fig. 5. A, The inhibitory action of furosemide (*Furo*) was independent of the membrane potential. Neurons were held at −60 mV and then at +50 mV. At each holding potential, NMDA and NMDA plus furosemide were applied sequentially three times. Records are the averages. B, Linear summation of Zn^{2+} and furosemide for inhibition of the NMDA-induced currents. The degree of inhibition by 20 μM Zn^{2+} was evaluated on control responses and on responses inhibited by furosemide. *Inset*, superimposition of responses to NMDA plus Zn^{2+} in the absence and in the presence of furosemide, once the furosemide-inhibited and uninhibited responses had been normalized for steady state amplitudes.

the right without changing the maximum inhibition. The IC_{50} value for niflumic acid was increased 1.6-fold and 3.6-fold in the presence of 0.5 and 1 mM spermine, respectively. The inhibitory action of furosemide was similarly attenuated by the inclusion of spermine (Fig. 6A).

Discussion

The present results demonstrate that responses to NMDA are inhibited by a class of compounds widely used clinically for various purposes. The common property of these compounds is that they are all inhibitors of anion transport systems. Furosemide and bumetanide are diuretics that act by inhibiting the active reabsorption of chloride in the loop of Henle (e.g., see Ref. 16). Niflumic and flufenamic acids are nonsteroidal anti-inflammatory agents that are potent noncompetitive inhibitors of the erythrocyte anion transporter (17, 18). With lower potency, these compounds are also inhibitors of a variety of anion channels, including those associated with the $GABA_A$ (19) and Ca^{2+}-dependent chloride channels (20, 21). However, it has not been shown previously that anion transport inhibitors are also able to reduce the activity of a cation channel-linked receptor like the NMDA receptor complex. Niflumic acid was the most potent compound in blocking the NMDA-induced current. The IC_{50} for niflumic acid as an inhibitor of the erythrocyte anion transporter is about 1 μM (16, 17). The concentration for half-maximal inhibition of NMDA-induced responses found in the present work is in the range of the values reported for inhibition of anion permeation through the $GABA_A$ receptor (30–155 μM) (19), whereas it is one order of magnitude higher than that for inhibition of the Ca^{2+}-dependent chloride channel of the *Xenopus* oocyte (27 μM) (21). Although blood levels attained at normal therapeutic doses of loop diuretics (estimated to be approximately, 30 μM for furosemide) should not seriously compromise brain NMDA receptor activity, the concentrations of these compounds used in several studies for inhibition of chloride fluxes in neurons (22, 23) and in *Xenopus* oocytes (24) proved to be active also at NMDA receptors. Conclusions from previous studies (e.g., see Ref. 24) are brought into question by the present findings.

The antagonism of NMDA-induced currents did not arise from the interaction with chloride permeation or from a modification of chloride equilibrium potential, because similar results were seen when methanesulfonate substituted for chloride as the main internal anion. Chloride transport inhibitors reduced the NMDA-induced current in a noncompetitive manner, because the degree of inhibition was similar in the range of 30–300 μM NMDA. Summation experiments involving Zn^{2+} and furosemide revealed no detectable interaction. Simultaneous application of furosemide (at a concentration above its IC_{50}) and Zn^{2+} (at a concentration adequate to block the NMDA response largely, by >80%) resulted in an inhibition that was the sum of inhibition induced by each alone. This result indicates that the chloride transport blockers act at a site independent from the Zn^{2+} binding site. Depression of NMDA-induced currents by this class of compounds was observed to

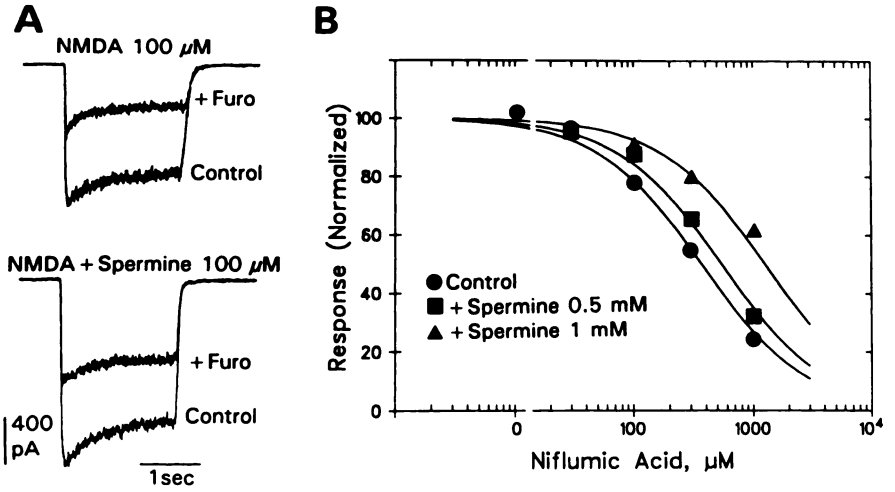

Fig. 6. Spermine potentiated the response to NMDA (plus 10 μM glycine) and decreased the inhibitory action of chloride transport inhibitors. A, Representative records (averaged, $n = 3$) showing the attenuation by 100 μM spermine of the inhibitory effect of furosemide (*Furo*) (2 mM). Note the increased current when spermine was present. B, Dose-inhibition curves for niflumic acid in the absence (control) and in the presence of 0.5 and 1 mM spermine. IC_{50} was shifted to 550 μM and 1.2 mM, respectively. *Points* are mean values obtained in three to seven cells for control, six cells with 0.5 mM spermine and four cells with 1 mM spermine. For clarity, the error bars have been omitted. Standard errors were <10%.

be equal at negative and positive inside potentials, i.e., when currents were flowing either in or out, indicating that binding of chloride transport inhibitors to the NMDA receptor complex did not sense the membrane electric field, making an interaction with the Mg^{2+} or phencyclidine binding sites unlikely.

The inhibitory action on NMDA-induced currents could not be surmounted by a 10-fold increase of glycine concentration. Inhibition induced by furosemide was, however, larger at non-saturating concentrations of glycine. This result is readily explained in terms of the antagonistic action that this class of compounds exerts on the polyamine modulatory site (see below). In binding experiments and in *Xenopus* oocytes, spermine increases the affinity of glycine for its receptor (13, 14). At a concentration of glycine of 0.3 μM ($\approx K_d$) (25), the glycine receptor occupancy should be about 50%. Considering the co-agonist nature of this amino acid (25, 26), a decrease in glycine affinity by antagonism of the polyamine site should appear as an additional reduction in sustained current and, therefore, a larger degree of inhibition. The idea that chloride transport inhibitors are not acting directly at the glycine receptor is supported by the fact that at saturating concentrations of glycine the induced inhibition became independent of the glycine concentration.

The polyamine spermine was able to antagonize the inhibitory action of niflumic acid and furosemide. Dose-inhibition curves for niflumic acid calculated in the absence and presence of spermine revealed a large decrease in the potency for inhibition of the NMDA-induced response when spermine was present. Assuming a simple model of allosteric potentiation of the NMDA receptor for polyamines (15), these results indicate that the inhibitors of chloride transport interact with a polyamine binding site. The fast on-off kinetics shown by these compounds suggest that the polyamine binding site(s) is located on the extracellular domain of the NMDA receptor. A few observations from concentration-jump experiments with spermine (τ_{on} and τ_{off}, <100 msec)[1] support this conclusion (see Ref. 13).[1] The presence of the polyamine was not necessary for antagonism to develop. This result may be explained in terms of inverse agonistic properties of anion transport blockers. Another interesting possibility is that inhibitors of chloride transport are agonists of a second (inhibitory) polyamine recognition site associated with the NMDA receptor complex (see Ref. 27 for review). Consistently with this suggestion, in preliminary experiments the inhibition induced by niflumic acid was not attenuated by inclusion of DET (150 μM), a competitive inhibitor of the polyamine potentiator site (15), at a concentration sufficient to totally abolish spermine potentiation. This would imply that the observed attenuation of chloride transport blocker inhibition by spermine, rather than being due to a competitive displacement of these compounds from the first (potentiator) binding site, actually arose from disclosure of the potentiation effect of polyamines on NMDA currents. Whatever the mechanism of action, these compounds proved useful for the control of activity at NMDA receptors independently of both the extracellular concentration of glutamate and/or glycine and the membrane potential.

An important issue raised by the present results is that chloride transport inhibitors may be useful drugs for preventing neuronal death produced during excitotoxic processes. Observations in cultured neurons revealed that exposure to the endogenous excitatory amino acid glutamate induces acute swelling, dependent on extracellular Na^+ and Cl^-, and delayed neuronal desintegration, dependent on extracellular Ca^{2+} (see Ref. 6 for review). Compounds preventing these two steps of excitoxicity should be highly efficient in preventing neuronal degeneration. Chloride transport inhibitors seem to have the double action of blocking chloride fluxes, therefore reducing swelling, and of attenuating NMDA receptor activation, therefore reducing the Ca^{2+} accumulation inside the cell. Recent experiments have shown total protection from glutamate-induced cell death by amiloride, a diuretic that is also active at NMDA receptors (28), and there are accumulating data indicating a protective effect of chloride transport blockers in neuronal degeneration (29–31). Although this dual mechanism of action may be helpful for therapeutic treatment of events leading to neuronal death, chemical modification to increase the affinity of chloride transport blockers for the polyamine regulatory site(s) is required for therapeutic usefulness.

Acknowledgments

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