Guanidino Compounds That Are Increased in Cerebrospinal Fluid and Brain of Uremic Patients Inhibit GABA and Glycine Responses on Mouse Neurons in Cell Culture

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Four guanidino compounds that have been found to be markedly increased in cerebrospinal fluid and brain tissue of uremic patients, namely, guanidine, methylguanidine, creatinine, and guanidinosuccinic acid, were applied to mouse spinal cord neurons in primary dissociated cell culture to evaluate their effects on postsynaptic responses to gamma-aminobutyric acid (GABA) and glycine. Intracellular microelectrode recording techniques were used. Guanidine, methylguanidine, creatinine, and guanidinosuccinic acid reversibly and in a dose-dependent manner inhibited both GABA and glycine responses. Guanidinosuccinic acid was the most potent inhibitor of the amino acid responses, followed in decreasing potency by methylguanidine, guanidine, and creatinine. Guanidinosuccinic acid inhibited responses to GABA and glycine, at concentrations similar to those found in cerebrospinal fluid and brain tissue of patients with terminal renal insufficiency. The other guanidino compounds tested exerted their effects only at concentrations higher than those found in uremic biological fluids and tissues. The inhibitory effect of guanidine and methylguanidine on responses to GABA was additive. The effect of the guanidino compounds on GABA responses was not antagonized by coapplication of the benzodiazepine-receptor antagonist CGS 9896. The results suggest that guanidine, methylguanidine, creatinine, and guanidinosuccinic acid inhibited responses to the inhibitory neurotransmitters GABA and glycine by blocking the chloride channel. The observed action of the studied guanidino compounds might contribute to the pathogenesis of the complex neurological symptomatology encountered in uremia.

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Patients with renal failure develop a variety of neurological symptoms and signs. Frequent neurological complications of uremia include encephalopathy, polyneuropathy, and epilepsy [1–3]. A number of abnormal movements, including muscle fasciculations, asterixis, and myoclonus, may occur in association with varying degrees of encephalopathy in patients with acute renal failure [1, 4, 5]. The basis for the majority of the neurological complications is uncertain, and the pathophysiological mechanisms still remain to be elucidated.

Guanidino compounds have been implicated as uremic toxins since they were found to be increased in uremic biological fluids [6–10] and brain [11] and were demonstrated to induce toxic effects. Guanidinosuccinic acid is suggested to be related to the uremic bleeding diathesis [12, 13] and has been shown to decrease erythrocyte transketolase activity [14]. Methylguanidine could be related to the uremic polyneuropathy [15] and has been shown experimentally to induce epilepsy [16] and a syndrome similar to the uremic twitch-convulsive syndrome [15, 17]. When injected intracisternally, methylguanidine, taurocyamine, homoarginine, creatine, and creatinine were found to have a convulsive effect in animals [18–22]. Applied on the sensorimotor cortex in rabbit, α-keto-δ-guanidinovaleric acid induced electroencephalographic epileptiform discharges [23]. Furthermore, methylguanidine inhibited brain sodium-potassium ATPase [24, 25].

In patients with severe renal insufficiency, the levels of guanidinosuccinic acid, creatinine, guanidine, and

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methylguanidine in serum as well as in cerebrospinal fluid were found to be at least ten times higher than in control subjects [10]. The same guanidino compounds were found to be increased from 5- to 100-fold in different brain regions of uremic subjects [11]. The experimentally observed toxicity of these guanidino compounds might contribute to the neurological symptomatology presenting in uremia. A reduction in gamma-aminobutyric acid (GABA)ergic inhibition in the central nervous system has been suggested to be a cause of epilepsy [26–29]. A series of convulsants have been shown to inhibit responses to iontophoretically applied GABA on mammalian spinal cord neurons grown in cell culture [30, 31]. Strychnine has been suggested to induce myoclonus, and at high concentrations convulsions, by blocking the synaptic action of the inhibitory amino acid transmitter glycine (GLY) [32], by interacting with the postsynaptic GLY receptor [33]. Furthermore, penicillin, known to inhibit GABA responses through an interaction with the GABA-receptor complex, induced spinal myoclonus after focal application in a spinal preparation in cat [34].

To determine the mechanisms through which guanidinosuccinic acid, creatinine, guanidine, and methylguanidine might produce seizures and myoclonus in uremia, we evaluated the effects of these guanidino compounds on responses evoked by iontophoretically applied GABA and GLY recorded from spinal cord neurons in cell culture.

Materials and Methods

Primary Dissociated Cell Culture

Cultures of spinal cord neurons were prepared from dissected spinal cords and attached dorsal root ganglia from 12- to 14-day-old fetal mice as described previously [35]. Experiments conformed to the policy of the American Physiology Society. The tissue was minced and then mechanically dissociated by trituration in Ca^{++}- and Mg^{++}-free balanced salt solution to a suspension of single cells and small clumps. The dissociated cells were suspended in culture medium (90% Eagle's minimal essential medium supplemented with 5.5 gm of glucose and 1.5 gm of sodium bicarbonate ($NaHCO_3$)/l, 5% heat-inactivated horse serum, and 5% Nu-Serum II [Collaborative Research, Inc.], 325 mOsmol) and then plated on sterile collagen-coated 35-mm dishes. The cultures were maintained in an incubator with an atmosphere of 93% room air and 7% carbon dioxide (CO_2) at 35°C. The bicarbonate/CO^2 buffer maintained the pH at 7.4. On days 4 to 6, 5-fluoro-2'deoxyuridine was added to the cultures to suppress the growth of rapidly dividing nonneuronal cells. Cultures were maintained for 4 to 9 weeks before electrophysiological experiments were performed.

Experimental Procedures

SOLUTIONS. All recordings were made in Dulbecco's phosphate-buffered saline (DPBS) solution with a high magnesium-ion concentration after removal of growth medium. The elevated magnesium concentration in the recording solution suppressed spontaneous synaptic and action potentials. The recording solution contained in mM: sodium chloride (NaCl), 137; sodium phosphate, dibasic (Na_2HPO_4), 8.06; potassium chloride (KCl), 2.68; potassium phosphate, monobasic (KH_2PO_4), 1.47; calcium chloride ($CaCl_2$), 1, magnesium chloride ($MgCl_2$), 10; and glucose, 5.6 (pH 7.4). Heavy paraffin oil was applied to the surface of the bathing solution to retard evaporation. Solutions of drugs were always prepared on the day of the experiment in the following manner: guanidine hydrochloride, methylguanidine hydrochloride, creatinine, and guanidinosuccinic acid were dissolved in DPBS to form 100 mM stock solutions. Aliquots were removed and diluted in bathing medium to obtain the applied concentrations. For the drug concentrations of 10 mM and 20 mM, iso-osmolar solutions were prepared by substituting sodium chloride with the respective guanidino compound. The pH of the solutions was 7.2 to 7.4.

Guanidine and methylguanidine were applied at concentrations between 100 nM and 100 mM. Creatinine concentrations ranged from 1 μM to 20 mM and guanidinosuccinic acid was tested at concentrations ranging between 10 μM and 100 mM. CGS 9896, a pyrazoloquinoline previously shown to be a pure benzodiazepine-receptor antagonist, was dissolved in dimethylsulfoxide to obtain a 10 mM stock solution. Aliquots were removed and diluted in bathing medium to obtain the applied concentration containing less than 0.1% dimethylsulfoxide.

EXPERIMENTAL APPARATUS. For experiments, the culture dish containing bathing solution was placed on a microscope fitted with phase contrast optics to facilitate micropipette placement (using micromanipulators) and to penetrate cells under direct visual control.

ELECTROPHYSIOLOGICAL RECORDINGS. Intracellular recordings were made from somata of spinal cord (> 20 μm) neurons using glass micropipettes (25 to 50 MΩ) filled with 3 M KCl. Use of an active bridge circuit (Model 8100, Dagan Corporation, Minneapolis, MN) allowed simultaneous recording of membrane potential and injection of current (for steady-state polarization or periodic stimulation) using a single micropipette. The preamplifier output signal was led to a six-channel polygraph (Model 2600, Gould Instruments Inc., Cleveland, OH) for continuous recording.

GABA AND GLYCINE RESPONSES. GABA (0.5 M, pH 3.4) and GLY (0.5 M, pH 3.0) were applied iontophoretically using 500-millisecond rectangular current pulses at 5-second intervals. Tips of iontophoretic pipettes were positioned to within 2 μm of neuronal somata. The use of 3 M KCl–filled micropipettes resulted in elevation of intracellular chloride concentration and a shift in the chloride equilibrium potential from about −65 mV to about −20 mV. Under these conditions, an increase in chloride conductance results in an outward chloride current [36] giving depolarizing GABA and GLY responses [36, 37]. Responses of about 10 to 15 mV in amplitude were evoked following membrane hyperpolarization to between 80 mV and 90 mV. Effects on GABA and GLY responses were accepted only if the re-

sponses returned to control amplitude within 2 minutes of removal of the drug-containing micropipette.

DRUG APPLICATION. For evaluation of drug effects on GABA and GLY responses, all guanidino compounds were applied by perfusion micropipette. A blunt tipped (10- to 15-μm) micropipette, filled with the recording solution containing the guanidino compound, was positioned 15 to 30 μm from the soma of the cell under study. The open end of the perfusion micropipette was connected to a pressure regulator, set between 0.4 and 0.8 psi, by tight-fitting polyethylene tubing. Pressure pulse duration, regulated by a voltage-activated three-way valve, was 10 seconds. Under these conditions, local perfusion produced no artifacts and application of recording solution (with or without vehicle) was virtually free of effects. During study of the effect of coapplication of the guanidino compounds and the benzodiazepine-receptor antagonist CGS 9896 [38], the drugs were applied through one perfusion micropipette to avoid flow artifacts. The perfusion micropipettes were held by Leitz micromanipulators. To decrease leakage of drugs into the bathing medium, the tips of the perfusion micropipettes were kept in the oil phase between drug application trials.

Drugs

GABA and the guanidino compounds guanidine, methylguanidine, creatinine, and guanidinosuccinic acid (Fig 1) were purchased from Sigma Chemical (St. Louis, MO). CGS 9896 (2-(*p*-chlorophenyl)-2,5-dihydropyrazolo)4,3-C(quinolin-3(5H)-one) was obtained from Ciba-Geigy (Summit, NJ).

Algebraic and Statistical Methods

At all applied concentrations, mean values and standard deviations were calculated for the effects on GABA and GLY responses. All effects were expressed as percent change from control GABA response. The statistical significance of differences between GABA and GLY responses with and without drug application was calculated using the two-tailed Student's *t* test; a *p* value of less than 0.05 was considered statistically significant.

Results

Direct Effects of Guanidine, Methylguanidine, Creatinine, Guanidinosuccinic Acid and CGS 9896 on GABA Responses

Application of the studied guanidino compounds at concentrations ranging from 100 nM to 20 mM to spinal cord neurons did not alter resting membrane potential or conductance. Guanidine and methylguanidine, applied at a concentration of 100 mM, induced a membrane depolarization of 10 to 25 mV. Application of recording solution (n = 16) did not significantly alter GABA responses (0.1 ± 1.7%). Application of CGS 9896 1 μM (n = 11) did not result in significant effects on GABA responses (0.23 ± 4.36% increase). Guanidine, methylguanidine, creatinine, and guanidinosuccinic acid rapidly and reversibly reduced GABA responses (Fig 2). The guanidino

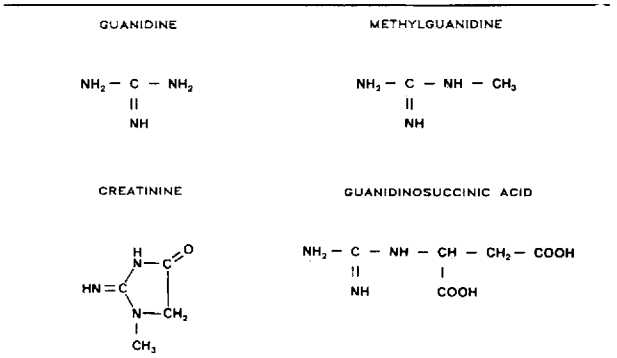

Fig 1. Structural formulas of the guanidino compounds guanidine, methylguanidine, creatinine, and guanidinosuccinic acid.

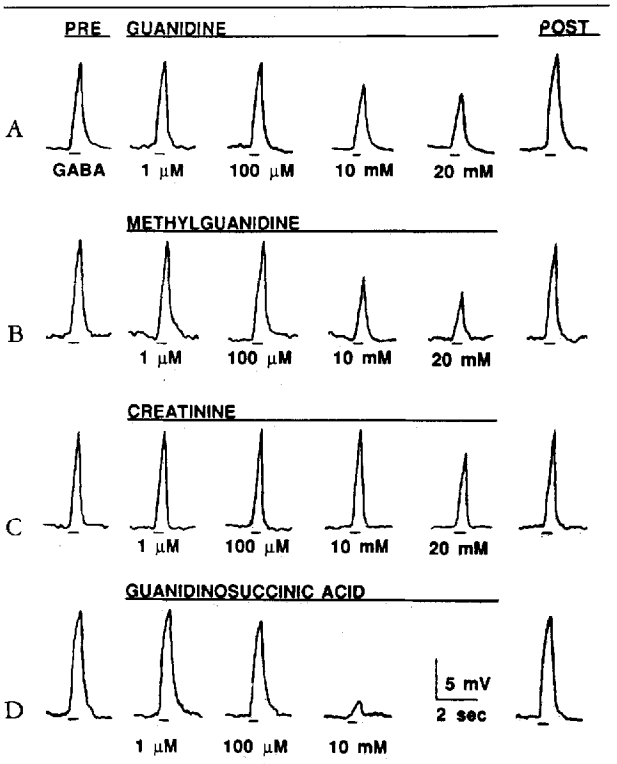

Fig 2. Reversible, concentration-dependent effects of guanidine (A), methylguanidine (B), creatinine (C), and guanidinosuccinic acid (D) on GABA responses on spinal cord neurons in primary dissociated cell culture. PRE shows stable GABA responses before drug application. The four middle responses show the effect of the guanidino compound. GABA responses returned to control values (POST) within 2 minutes following removal of the guanidino compound–containing micropipette. Iontophoretic application of GABA is indicated with a dash.

compound–induced effects were concentration dependent (Fig 3). A significant 23.9 ± 8.45% decrease ($p < 0.01$) (n = 10) of GABA responses was obtained with 10 mM guanidine and complete inhibition was observed with 100 mM (n = 5). A significant decrease of 34.7 ± 14.5% ($p < 0.001$) (n = 16) was obtained

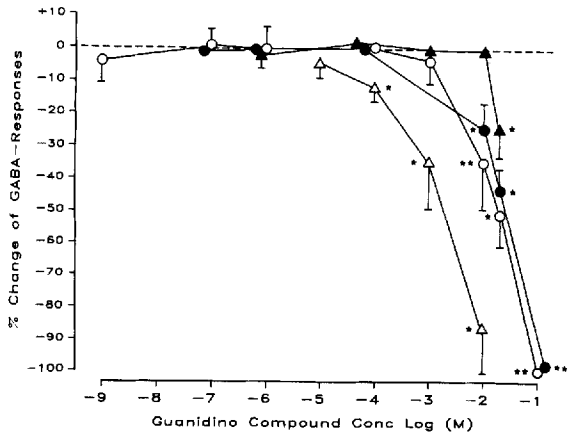

Fig 3. Concentration-dependent change of GABA responses in spinal cord neurons by guanidine (black circles), *methylguanidine* (white circles), *creatinine* (black triangles), *and guanidinosuccinic acid* (white triangles). *Effects are expressed in percent decrease of the original GABA response. Data shown are mean values and standard deviations. Four to sixteen cells were studied for each concentration tested. Asterisk indicates* $p < 0.01$, *double asterisk,* $p < 0.001$ *from control GABA response. Guanidino compound concentrations on the abscissa are logarithm molar.*

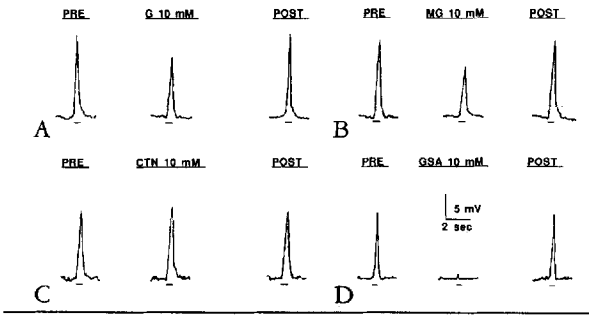

Fig 4. Reversible effects of (A) guanidine (G) (10 mM), (B) methylguanidine (MG) (10 mM), (C) creatinine (CTN) (10 mM), and (D) guanidinosuccinic acid (GSA) (10 mM) on glycine responses on spinal cord neurons. PRE shows stable glycine responses before drug application. The middle response shows the effect of the miniperfused drug. Glycine responses returned to control values (POST) within 2 minutes following removal of the guanidino compound–containing micropipette. Iontophoretic application of glycine is indicated with a dash.

at 10 mM methylguanidine and a total reduction of GABA responses was observed at 100 mM methylguanidine (n = 5). Creatinine was devoid of any significant effect on GABA responses at concentrations up to 10 mM. At 20 mM, however, creatinine significantly reduced GABA responses by 23.5 ± 7.55% ($p < 0.01$) (n = 9). The weak inhibition of GABA responses (3.72 ± 4.76%) (n = 6) by guanidinosuccinic acid applied at 10 μM was not statistically significant. However, a significant 12.1 ± 3.69% guanidinosuccinic acid–induced decrease ($p < 0.01$) (n = 6) of GABA responses was obtained at 100 μM and an almost complete inhibition of GABA responses (85.9 ± 13.5%) (n = 5) was observed at 10 mM. Guanidinosuccinic acid was approximately ten times more potent than guanidine and methylguanidine in inhibiting GABA responses.

Direct Effects of Guanidine, Methylguanidine, Creatinine, and Guanidinosuccinic Acid on Glycine Responses

Guanidine (10 mM), methylguanidine (10 mM), creatinine (20 mM), and guanidinosuccinic acid (100 μM and 10 mM) rapidly and reversibly decreased GLY responses in a statistically significant manner ($p < 0.01$) (Fig 4, Table 1). The guanidino compounds were equally potent in decreasing GLY and GABA responses.

Table 1. Effects of Guanidine, Methylguanidine, Creatinine, and Guanidinosuccinic Acid on Glycine (GLY) Responses on Mouse Spinal Cord Neurons

		No. of Cells Studied	GLY Responses % Decrease (Mean ± SD)
Guanidine	10 mM	5	28.1 ± 6.31[a]
Methylguanidine	10 mM	5	28.1 ± 8.52[a]
Creatinine	10 mM	5	1.86 ± 2.68
	20 mM	6	31.0 ± 7.77[a]
Guanidino-succinic acid	100 μM	4	14.8 ± 6.00[a]
	10 mM	9	96.6 ± 6.45[a]

[a] $p < 0.01$, from control, Student's two-tailed t test.

Effects of Coapplication of Guanidine, Methylguanidine, Creatinine, and Guanidinosuccinic Acid with CGS 9896 on GABA Responses

The effects on GABA responses of guanidine (10 mM), methylguanidine (10 mM), creatinine (20 mM), and guanidinosuccinic acid (10 mM) alone or in combination with CGS 9896 (1 μM), a pure benzodiazepine-receptor antagonist, were not significantly different (Table 2).

Additive Effects of Guanidine and Methylguanidine on GABA Responses

Coapplication of methylguanidine (10 mM) and guanidine (10 mM) resulted in a significantly larger inhibition of GABA responses than when either of these compounds were applied alone at 10 mM. Whereas methylguanidine (10 mM) reduced GABA responses 25.7 ± 7.37% (n = 3) and guanidine (10 mM) reduced GABA responses 23.3 ± 4.1% (n = 3), their

Table 2. Effect of CGS 9896, Guanidine, Methylguanidine, Creatinine, and Guanidinosuccinic Acid on GABA Responses on Mouse Spinal Cord

	No. of Cells Studied[a]	GABA Responses % Decrease (Mean ± SD)
CGS 9896 (1 μM)	11	0.23 ± 4.36
Guanidine (10 mM)	3	24.9 ± 0.75
Guanidine (10 mM) + CGS 9896 (1 μM)	3	24.3 ± 9.10
Methylguanidine (10 mM)	5	28.5 ± 3.69
Methylguanidine (10 mM) + CGS 9896 (1 μM)	5	32.2 ± 6.74
Creatinine (20 mM)	3	21.0 ± 5.81
Creatinine (20 mM) + CGS 9896 (1 μM)	3	21.1 ± 7.39
Guanidinosuccinic acid (10 mM)	3	94.7 ± 9.12
Guanidinosuccinic acid (10 mM) + CGS 9896 (1 μM)	3	93.3 ± 11.5

[a]Vertical lines indicate paired samples. No significant differences were found (Student's *t* test for paired samples).

coapplication resulted in a 39.7 ± 7.93% (n = 3) decrease of GABA responses.

Discussion

A variety of convulsants have been shown previously to inhibit GABAergic inhibition [37, 39–41] through interaction with the postsynaptic GABA-receptor complex consisting of a functionally coupled benzodiazepine receptor, GABA receptor, and chloride channel [42, 43]. Several convulsants reduced responses to iontophoretically applied GABA on mouse neurons grown in cell culture. DMCM, a convulsant beta-carboline, and CGS 8216, a pyrazolo-quinoline with proconvulsant effect, inhibited GABA responses [38, 41, 44, 45] through an interaction with the benzodiazepine receptor. Moreover, the convulsants bicuculline, picrotoxin, and pentylenetetrazole were previously reported to selectively antagonize GABA-mediated postsynaptic inhibition in cultured mammalian neurons [37, 39, 41, 45]. Penicillin, a convulsant that is known to induce myoclonus after intravenous administration in humans [46] and focal application in cat, [34] also antagonized GABA responses on mouse neurons [39]. Strychnine, inducing myoclonus at low doses and convulsions at higher concentrations, has been shown previously to be a GLY-receptor antagonist [33].

In this study, we investigated the effects of four guanidino compounds, which were found to be increased in cerebrospinal fluid and brain of uremic patients [10], on responses to the iontophoretically applied inhibitory neurotransmitters GABA and GLY on mouse neurons in primary dissociated cell culture. The guanidino compounds were guanidine, methylguanidine, creatinine, and guanidinosuccinic acid.

Guanidine, methylguanidine, creatinine, and guanidinosuccinic acid inhibited both GABA and GLY responses on mouse spinal cord neurons in culture in a concentration-dependent manner. Guanidinosuccinic acid was the most potent compound, followed by methylguanidine, guanidine, and creatinine. Guanidinosuccinic acid inhibited GABA and GLY responses at concentrations similar to those found in cerebrospinal fluid (up to 32 μM) and brain tissue (up to 21 nmol/gm of tissue) of patients with terminal renal insufficiency. The studied guanidino compounds did not reduce GABA responses through an interaction with the benzodiazepine receptor. Indeed, CGS 9896, a pyrazoloquinoline and pure benzodiazepine-receptor antagonist [38], did not antagonize the guanidino compound–induced inhibition of GABA responses. Earlier studies on cultured neurons demonstrated that the inhibitory neurotransmitters GABA and GLY act through different receptors on the membrane surface [30, 47, 48]. Moreover, activation of chloride conductance has been demonstrated to underlie the GABA- and GLY-receptor-coupled events [49]. Since GABA and GLY exert their inhibitory effects by activation of chloride conductance through interaction with different receptors, our observations suggest that guanidine, methylguanidine, and guanidinosuccinic acid, shown here not to be benzodiazepine-receptor ligands, inhibit inhibitory amino acid–responses by blocking chloride channels.

Structure-activity relationships cannot be established from the data presented in this report. Creatinine, the anhydrized form of creatine, was the least potent in decreasing responses to GABA and GLY, whereas the monosubstituted guanidino compounds guanidinosuccinic acid and methylguanidine were more potent than guanidine. Previous reports demonstrated that the potency of *N*-alkyl-guanidino compounds in blocking ionic channels of the motor end-plate membrane was a function of the length of the alkyl side chain [50]. It was suggested that the blocking potency, enhanced by

lengthening the side chain, might be related to the increase in side chain hydrophobicity.

The observed inhibitory effects of guanidine, methylguanidine, creatinine, and guanidinosuccinic acid on inhibitory neurotransmitter responses might have pathophysiological implications in uremia. Indeed, guanidinosuccinic acid displayed significant effects on GABA and GLY responses at concentrations similar to those found in cerebrospinal fluid and brain tissue of uremic patients [10, 11]. Moreover, guanidine, methylguanidine, and creatinine, which reduced GABA and GLY responses only at concentrations higher than those hitherto found in cerebrospinal fluid of uremic patients, might have, perhaps in combination with still other toxins, additive effects. The additive inhibitory effect of guanidine and methylguanidine on responses to GABA has been demonstrated in this report. A summative effect has also been shown for creatinine, creatine, guanidinoacetic acid, and guanidine in an experimental paradigm testing in vitro autohemolysis [24]. Earlier reports demonstrated methylguanidine [16] to be more potent than creatinine [20] in inducing seizures in rabbit after intracisternal administration. This is in agreement with the higher potency of methylguanidine in decreasing responses to inhibitory neurotransmitters as illustrated in this report. The in vivo epileptogenicity of guanidine and guanidinosuccinic acid has not been demonstrated or disproved as yet.

In addition to reducing GABA and GLY responses, other postsynaptic and presynaptic effects have been shown for guanidino compounds in the peripheral as well as in the central nervous system. Guanidine has been shown to increase transmitter release at the frog, rat, and crayfish neuromuscular junctions [51–54] and in the guinea pig olfactory cortex [55]. In addition, methylguanidine inhibited responses to acetylcholine in frog sartorious muscle [50, 52] and guanidine was found to have a dual effect on cat spinal monosynaptic reflex transmission (inhibiting at low doses and enhancing at high doses) [56]. These electrophysiological effects and reduction of GABA and GLY responses by the guanidino compounds might contribute to the central and peripheral nervous system symptomatology presenting in uremia. Moreover, methylguanidine has been shown to inhibit brain sodium-potassium ATPase, an effect which could also contribute to its epileptogenic activity [25].

In conclusion, guanidine, methylguanidine, creatinine, and guanidinosuccinic acid, found to be increased in cerebrospinal fluid and brain of uremic patients, inhibited responses to the inhibitory neurotransmitters GABA and GLY on mouse neurons in cell culture. Guanidinosuccinic acid inhibited GABA and GLY responses at concentrations similar to those previously found in cerebrospinal fluid and brain of uremic patients. The underlying mechanism is suggested to be the blocking of the chloride channel. This effect, alone or in combination with other effects exerted by these compounds or other toxins, might underlie the pathogenesis of the myoclonus, epilepsy, and encephalopathy presenting in uremia.

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