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Activation of $GABA_A$ Receptors by Guanidinoacetate: A Novel Pathophysiological Mechanism

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Guanidinoacetate methyltransferase (GAMT) deficiency is an autosomal recessively inherited disorder of creatine biosynthesis. The disease occurs in early life with developmental delay or arrest and several neurological symptoms, e.g., seizures and dyskinesia. Both the deficiency of high-energy phosphates in neurons and the neurotoxic action of the accumulated metabolite guanidinoacetate (GAA) are candidate mechanisms for the pathophysiology of this disease. To examine a potential role of GAA accumulation, we analyzed the electrophysiological responses of neurons induced by GAA application in primary culture and acute murine brain slices. GAA evoked picrotoxin- and bicuculline-sensitive $GABA_A$ receptor-mediated chloride currents with an EC_{50} of 167 μM in cortical neurons. Pathophysiologically relevant GAA concentrations hyperpolarized globus pallidus neurons and reduced their spontaneous spike frequency with an EC_{50} of 15.1 μM. Furthermore, GAA acted as a partial agonist at heterologously expressed $GABA_A$ but not $GABA_B$ receptors. The interaction of GAA with neuronal $GABA_A$ receptors represents a candidate mechanism explaining neurological dysfunction in GAMT deficiency. © 2002 Elsevier Science (USA)

Key Words: **creatine deficiency; guanidino compounds; globus pallidus; GABA receptor agonist.**

INTRODUCTION

Guanidinoacetate methyltransferase (GAMT) deficiency is an autosomal recessively inherited disorder of creatine biosynthesis (Stöckler *et al.,* 1994, 1996b; von Figura *et al.,* 2001). GAMT deficiency manifests during the first months of life as a developmental delay or arrest. Neurological symptoms are heterogeneous, including muscular hypotonia and weakness, poor head control, involuntary extrapyramidal movements, epilepsy, and autistic or self-aggressive behavior in older patients (Ganesan *et al.,* 1997; Leuzzi *et al.,* 2000; Schulze *et al.,* 1997; Stöckler *et al.,* 1994, 1996b; von Figura *et al.,* 2001). Abnormal signal intensities in the globus pallidus, as observed on magnetic resonance imaging (MRI) scans, may provide an important clue for diagnosis. The diagnosis of the disease is based on the detection of excessive amounts of guanidinoacetate (GAA) in urine, serum, and cerebrospinal fluid (CSF), the deficiency of creatine / phosphocreatine in brain magnetic resonance spectroscopy, and the absence of GAMT activity in fibroblasts or lymphocytes (Ilas *et al.,* 2000; Leuzzi *et al.,* 2000; Stöckler *et al.,* 1994, 1997).

Creatine is synthesized in a two-step mechanism from glycine, arginine, and methionine. In a first rate-limiting reaction, GAA and ornithine are formed by arginine:glycine amidinotransferase. In a second reaction, catalyzed by GAMT, *S*-adenosylmethionine do-

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nates the methyl group for formation of creatine from GAA (Walker, 1979). In cells with a highly fluctuating energy demand such as in muscle and brain, the creatine/phosphocreatine system is thought to serve as a temporal and spatial energy buffering system. Oral supplementation of creatine in GAMT-deficient patients partially restores the creatine/phosphocreatine in brain and has beneficial effects on the clinical manifestations of some patients (Ganesan *et al.*, 1997; Leuzzi *et al.*, 2000; Stöckler *et al.*, 1996a). A dietary therapeutic approach to decrease GAA levels further improved the symptoms in one patient (Schulze *et al.*, 1998).

The potential neurotoxic actions of several guanidino compounds, among them several substances accumulating in uremia, were studied in recent reports and suggested possible interactions with the $GABA_A$ receptor (D'Hooge *et al.*, 1999; De Deyn *et al.*, 1991). These studies and the severe neurological symptoms observed in patients with GAMT deficiency prompted us to examine the electrophysiological effects of GAA on cultured neocortical mouse neurons and globus pallidus neurons of acute murine brain slices.

Here, we show that pathophysiologically relevant concentrations of GAA activate a chloride conductance via $GABA_A$ receptors and significantly alter spontaneous electrical activity of cultured neurons and of neurons in *in vitro* brain slices. The interaction of accumulated GAA with $GABA_A$ receptors might represent a molecular mechanism that induces neurological dysfunction in GAMT-deficient patients.

MATERIALS AND METHODS

Cell Culture and Slice Preparation

Cortex neurons of neonatal C57Bl/6J mice were prepared and cultured as described (Neuhoff *et al.*, 1999) and used after 10 days in culture. For patch-clamp recordings, glass coverslips containing neurons were transferred to dishes continuously perfused at 2–4 ml/min with extracellular solution (ES, see below). Coronal brain slices (250 μm) from C57Bl/6J mice (10–13 days old, killed by cervical dislocation) were prepared as previously described (Liss *et al.*, 1999). For patch-clamp recordings, slices were transferred to a chamber continuously perfused at 2–4 ml/min with artificial cerebrospinal fluid (ACSF) bubbled with a mixture of 95% O_2, 5% CO_2 at room temperature, and allowed to recover for 30 min.

Patch-Clamp Recordings

Recordings were carried out with an EPC9 patch-clamp amplifier, and the program package Pulse + Pulsefit was used for data acquisition (HEKA Electronic, Germany). Patch pipettes were pulled from borosilicate glass using a DMZ puller (Zeitz, Germany). Series resistances ranged from 2 to 3 MΩ for voltage-clamp recordings and 3 to 7 MΩ for current-clamp recordings, and were electronically compensated (60–80%). Recordings were digitized at 2–5 kHz and filtered with low-pass filter Bessel characteristics of 0.4–1 kHz cutoff frequency.

ES contained (in mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 Hepes, 10 glucose, 20 sucrose, pH 7.4 (adjusted with NaOH). For voltage-clamp experiments, 0.5 μM tetrodotoxin (TTX) was added to ES. ACSF contained (in mM) 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 glucose. Intracellular solution contained (in mM) 120 K-gluconate, 20 KCl, 10 Hepes, 10 EGTA, 2 $MgCl_2$, 2 ATP, pH 7.3 (adjusted with KOH) unless otherwise stated. To obtain different intracellular chloride concentrations, the relative amounts of K-gluconate and KCl were adjusted. For experiments with $GABA_B$ receptors, 0.2 mM GTP was added to the internal solution.

Experiments were performed at room temperature (20°C). The Nernst equation was used to calculate the reversal potential for chloride:

$$E_{Cl} = -58.17 * \log([Cl^-]_{ext}/[Cl^-]_{int}).$$

For rapid application of substances, a DAD12 microapplicator (A&L Associates, U.S.A.) was used. In all other experiments substances were applied by a gravity-driven perfusion system.

Igor Pro (Wavemetrics, U.S.A.) and Excel 7 (Microsoft, U.S.A.) were used for data analysis and graphics. Dose–response curves were fitted with the following Hill functions:

$$I(C) = I_{max}(1 - 1/(1 + (C/EC_{50})^H))$$

for voltage-clamp,

$$f(C)/f_{max} = 1/(1 + (C/EC_{50})^H)$$

for current-clamp experiments.

Data are given as means ± SEM. For statistical comparison in Fig. 4D, a two-sided *t*-test (Excel 7) was used.

Functional [^{35}S]TBPS Ligand Autoradiography

We used a previously described procedure (Korpi *et al.*, 1996) to analyze the effect of GAA on TBPS binding. Briefly, 20-μm-thick coronal brain sections were cut with a cryostat, mounted on alkyl silane-covered glass slides, and preincubated on ice three times for 10 min in 50 mM Tris (pH 7.4) containing 1 mM EDTA. Incubation of sections with [^{35}S]TBPS (600 dpm/μl (PerkinElmer Life Sciences, Cambridge, UK) adjusted with nonradioactive TBPS to 6 nM) was performed in a humid chamber for 90 min at 22°C with an incubation buffer containing 50 mM Tris (pH 7.4) and 120 mM NaCl. In Figs. 5B and 5C, the buffer was supplemented with 2 μM GABA, 100 μM GAA, or 300 μM GAA. The sections were subsequently washed three times for 15 s in ice-cold incubation buffer, dipped into distilled water, and air-dried. The slides were exposed at −70°C to Hyperfilm Bio-Max MS (Amersham Biosciences, Freiburg, Germany) for 5–7 days. All chemicals of the highest available purity were from Sigma (Munich, Germany) unless otherwise stated.

Expression of Recombinant $GABA_A$ Receptors in Xenopus Oocytes

The cDNAs encoding the α1, β2, and γ2s subunits of the rat $GABA_A$ receptor channel (Malherbe *et al.*, 1990) were inserted into the polylinker of the pBC/CMV vector. This expression vector allows high-level expression of a foreign gene under control of the cytomegalovirus promoter. A 1:1:1 (w:w:w) plasmid mixture of α1, β2, and γ2s was diluted (45 ng/μl) in a buffer containing 88 mM NaCl, 1 mM KCl, and 15 mM Hepes (pH 7.0) for intranuclear oocyte injection. Oocytes of maturation stages V to VI of South African frogs (*Xenopus laevis*) were used for expression. About 0.4 ng of the cDNA mixture was injected into the nuclei of the oocytes as described (Bertrand *et al.*, 1991). During the experiments on the following 2 days, the oocytes were superfused by a solution containing (in mM) NaCl 90, KCl 1, $MgCl_2$ 1, $CaCl_2$ 1, Hepes 5 (pH 7.4, room temperature). The membrane potential was held at −80 mV by a 2-microelectrode voltage-clamp circuit (TurboTec05, NPI electronic, Tamm, Germany), and GAA or GABA was applied every 2.5 min for 10 s.

Expression of Recombinant $GABA_B$ Receptors and GIRK Channels in Chinese Hamster Ovary (CHO) Cells

CHO cells stably expressing $GABA_B$R1a/$GABA_B$R2 receptor subunits (Wise *et al.*, 1999) were grown in Dulbecco's modified Eagle's Alpha medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) and antibiotics. Transfection of CHO cells with lipofectamine reagent (Invitrogen, Karlsruhe, Germany) was performed using 1 μg plasmid DNA encoding Kir3.1/Kir3.2 K channels and enhanced green fluorescent protein (EGFP). Transfected cells were identified by EGFP expression. K currents were recorded with a ramp voltage-clamp protocol from −120 to 0 mV on the first day after transfection at room temperature (22–25°C) using solutions listed above.

RESULTS

Application of Guanidinoacetate (GAA) Evokes Picrotoxin- and Bicuculline-Sensitive Chloride Currents in Cultured Neurons

To explore possible effects of GAA on neuronal electrical activity, we used dissociated, cultured mouse cortical neurons. The spontaneously active neurons formed robust synaptic connections that led to concerted network activity and had a resting membrane potential of −54.5 ± 1.2 mV (n = 15, Fig. 1A). Bath application of GAA at a concentration of 100 μM depolarized the cells by 13.5 ± 1.9 mV and inhibited neuronal network activity (Fig. 1B; V_m: −42.3 ± 1.3 mV, n = 5). The effect of GAA was fully reversible upon washout (Fig. 1B). In contrast, no change in membrane potential and/or spike frequency was observed when 1 mM creatine was bath applied (Fig. 1C; V_m: −53.0 ± 3.5 mV, n = 4). The GAA analogue guanidinopropionate (GPA, 100 μM) induced similar effects on membrane potential and spike frequency compared to GAA (Fig. 1D; V_m: −40.4 ± 3.1 mV, n = 6). Next, we determined the reversal potential of the GAA-induced current using a ramp voltage-clamp protocol from −80 to −20 mV (Fig. 2A) on TTX-treated neurons. Analysis of the difference current calculated from the currents determined before and after GAA application revealed the activation of a conductance with a reversal potential of −43.7 ± 0.6 mV (Fig. 2A, n = 5). The reversal potential of this GAA-induced current was very close to the calculated Nernst potential for chloride under the chosen experimental conditions (−45.8 mV; $[Cl^-]_{int}$ = 24 mM, $[Cl^-]_{ext}$ = 144 mM). This suggested that chloride ions

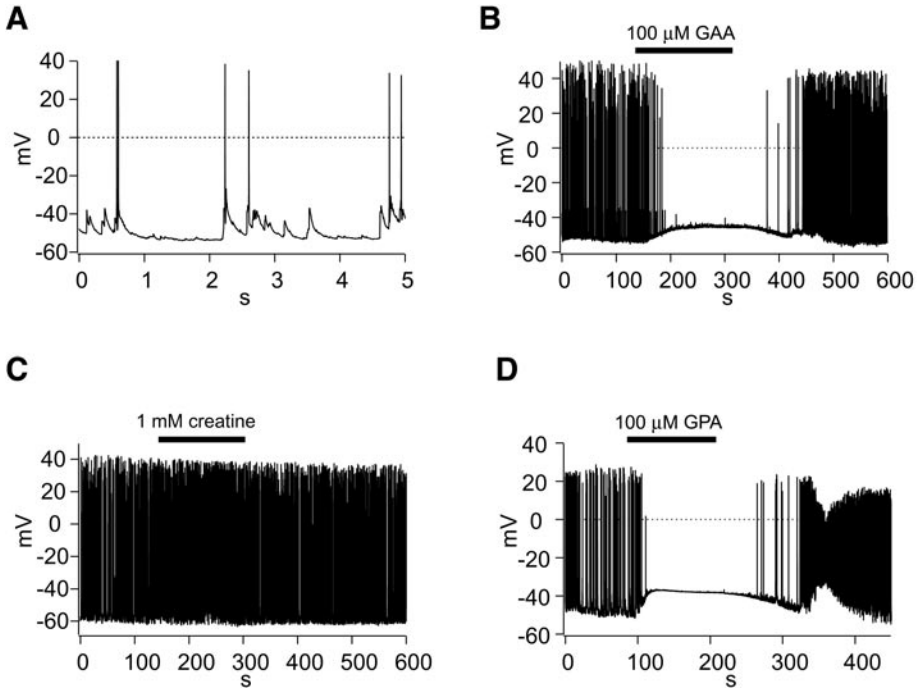

FIG. 1. Effects of guanidino compounds on cultured neurons. (A) Current-clamp recordings of cultured neurons show synaptic potentials and spontaneous spiking activity. (B) Application of the creatine precursor GAA (100 μM) depolarized neurons and stopped spontaneous spiking activity. These effects were reversible upon washout. (C) In contrast, creatine at concentrations of up to 1 mM had no effect on the resting potential and spiking activity. (D) Application of the GAA analogue GPA (100 μM) reversibly depolarized neurons and stopped spiking activity.

were the main charge carrier of the GAA-induced conductance. Increasing the internal chloride concentration indeed shifted the reversal potential of the GAA-induced current as expected for a highly selective chloride conductance (Fig. 2B; E_{rev}(mV): -56.6 ± 1.6 (14 mM, $n = 5$), -27.5 ± 2.0 (54 mM, $n = 5$), and -4.0 ± 0.9 (144 mM, $n = 3$)). We therefore concluded that GAA application activated voltage-gated or ligand-gated chloride channels and considered the possibility that, due to its structural similarity with GABA, GAA might act on neuronal $GABA_A$ receptors. Next, we applied the $GABA_A$ receptor antagonist picrotoxin (PTX, 50 μM) on TTX-treated neurons, which completely and reversibly blocked GAA-induced currents (Fig. 2C, $98.9 \pm 0.1\%$ block, $n = 4$), indicating an interaction of GAA with $GABA_A$ receptors.

We used a rapid application system to further characterize the GAA-induced chloride current. Application of 1 mM GAA induced large membrane currents (Fig. 3A; GAA: 5.42 ± 0.71 nA, $n = 5$) that quickly deactivated after removal of GAA. The GAA-induced currents were almost completely blocked ($95 \pm 1\%$, $n = 5$) by coapplication of 30 μM of the competitive $GABA_A$ receptor antagonist bicuculline (Fig. 3B). In contrast, creatine at concentrations of up to 1 mM did not evoke any membrane currents (Fig. 3C, $n = 5$). We therefore concluded that receptor activation might be critically dependent on the absence of the methyl group in GAA and GPA as compared to creatine (Fig. 3E).

In GAMT-deficient individuals, GAA is prevalent in concentrations up to levels of 12 μM in CSF and 30 μM in serum (Leuzzi *et al.*, 2000; Stöckler *et al.*, 1997). We used the GAA-induced currents of the rapid application experiments to construct a concentration–response curve for cultured neurons (Fig. 3D, inset), which was well described by a Hill function with an EC_{50} of 167 ± 40 μM and a Hill coefficient of 1.40 ± 0.04 (Fig. 3D, $n = 8$). Consequently, pathophysiologically relevant extracellular GAA concentrations between 10 and 30 μM evoked nonsaturating $GABA_A$ receptor currents of 171 ± 55 and 720 ± 198 pA, respectively.

GAA-Induced Inhibition of Spontaneous Electrical Activity of Globus Pallidus Neurons

MRI imaging studies identified the globus pallidus (GP) as a target of disease-associated changes in

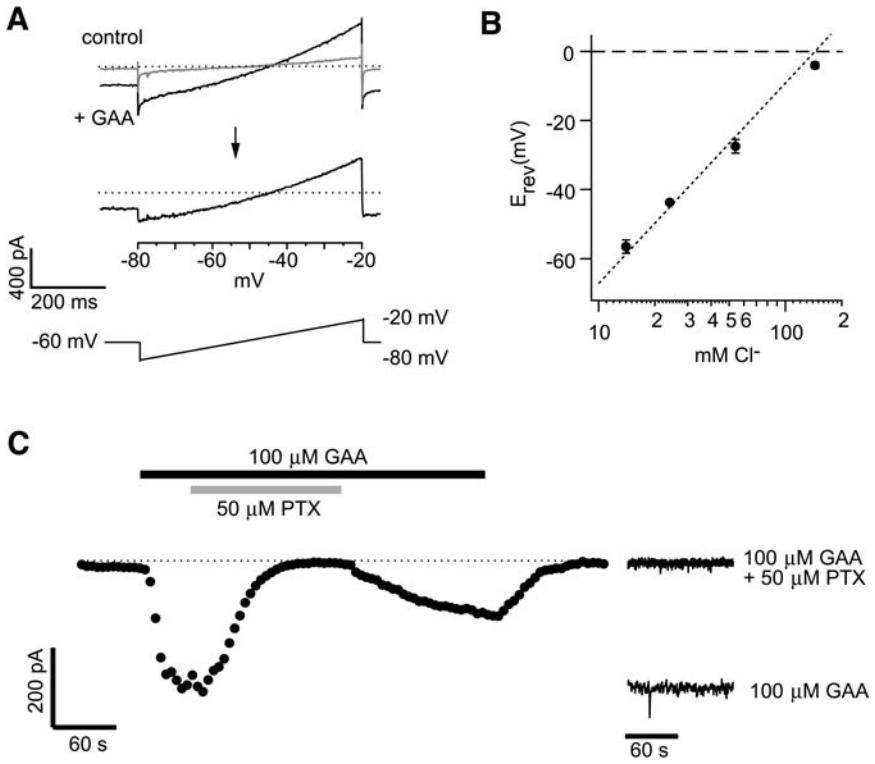

FIG. 2. GAA activates a chloride conductance. (A) Voltage-clamp recordings of cultured neurons in TTX (0.5 μM). Application of GAA (100 μM) changed current responses to a ramp protocol from −80 to −20 mV (upper trace). The net current was obtained by subtracting the control recording from the recording in GAA (bottom trace). The reversal potential of the GAA-induced current was −43.7 ± 0.6 mV, close to the calculated reversal potential for chloride according to the Nernst equation (−45.8 mV). (B) Exchanging the internal chloride concentration shifted the reversal potential as expected for a highly selective conductance. The dotted line represents the calculated shift of the chloride reversal potential. Internal chloride concentrations were 14, 24, 54, and 144 mM (n = 3–5). (C) Voltage-clamp recordings of a neuron held at −80 mV. Application of GAA (100 μM) induced an inward current that was blocked by coapplication of PTX (50 μM) and partially recovered after washout of PTX. Representative current traces recorded during application of GAA or GAA + PTX are shown on the right side.

GAMT deficiency (Ganesan *et al.*, 1997; Leuzzi *et al.*, 2000; Stöckler *et al.*, 1994). The GP is suspected to play a major role in the pathogenesis of pronounced extrapyramidal symptoms in GAMT deficiency (von Figura *et al.*, 2001). To allow a more direct assessment of the effect of GAA on this target structure, we carried out patch-clamp recordings of GP neurons in coronal mouse brain slices. In agreement with previous studies, GP neurons showed spontaneous discharges with a mean frequency of 10.0 ± 3.3 Hz (n = 35), thus resembling most closely type A GP neurons in rat (Cooper and Stanford, 2000).

We used a low-chloride internal pipette solution (4 mM) to simulate the negative physiological reversal potential of chloride in the mature mammalian brain. Upon superfusion with 1 mM GAA, GP neurons hyperpolarized to membrane potentials of −61.5 ± 1.8 mV (n = 5). The hyperpolarization attenuated the spontaneous spiking activity (Fig. 4A). After washout of GAA, the regular spiking of the neurons reappeared.

We next examined functional effects of clinically relevant concentrations of GAA. We used the spike frequency as a functional parameter, which can be correlated with the transmitter release in the axonal target areas. GAA concentrations of 10 and 30 μM induced significant reductions of spontaneous spiking frequencies in GP neurons to 71.6 ± 18.4% (n = 5) and 17.4 ± 13.2% (n = 7) of control conditions, respectively (Fig. 4B). The concentration–response curve of GAA was well described by a Hill function with an EC_{50} of 15.1 μM and a Hill coefficient of 2.3 (n = 5–23) (Fig. 4C). When we coapplied 100 μM GAA with 50 μM of the $GABA_A$ receptor antagonist PTX, the GAA-induced reduction of discharge frequencies was almost completely masked, indicating that, in accordance

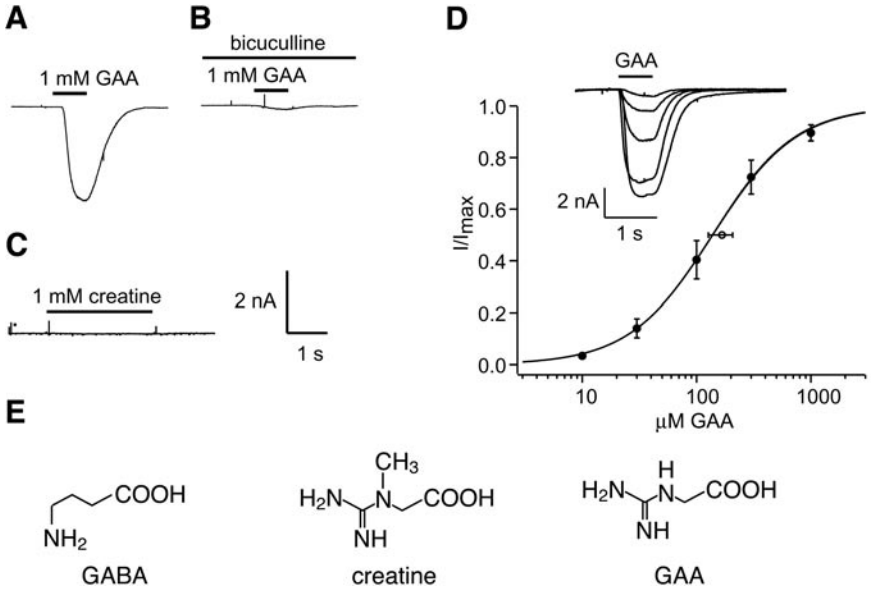

FIG. 3. GAA activates currents at pathophysiologically relevant concentrations. Rapid application of agonists on cultured neurons in TTX (0.5 μM) held at −60 mV. (A) Application of GAA (100 μM) induced inward currents that quickly deactivated following GAA washout. (B) GAA-induced currents were blocked by coapplication of the $GABA_A$ receptor antagonist bicuculline (30 μM). (C) Application of creatine (1 mM) had no effect on membrane currents. (D) The GAA-induced currents were dose dependent (see inset) and were fit by a Hill function with an IC_{50} of 167 ± 40 μM and a Hill coefficient of 1.4 ± 0.04. (E) The chemical structures of GABA, creatine, and GAA reveal a high degree of similarity. Please note the methyl group at position two of creatine that is absent in GAA and GABA.

with our findings in cultured neurons, GAA acts on GP neurons through activation of $GABA_A$ receptors (Figs. 4D and 4E; 100 μM GAA: 4.9 ± 4.7%; 100 μM GAA + 50 μM PTX: 84.4 ± 9.3%, $n = 7$).

GAA Reduces [^{35}S]Butylbicyclophosphorothionate (TBPS) Binding in Mouse Brain

We used functional [^{35}S]TBPS ligand autoradiography (Korpi *et al.*, 1996) to evaluate the effect of GAA application on [^{35}S]TBPS binding in mouse coronal brain sections. Upon binding of GABA-mimetics, [^{35}S]TBPS binding to $GABA_A$ receptors is inhibited. When we co-incubated brain sections with 100 or 300 μM GAA and [^{35}S]TBPS (Figs. 5C and 5D), we observed a reduction in the remaining radioligand binding as compared to the [^{35}S]TBPS control (Fig. 5A). However, the binding of [^{35}S]TBPS to native $GABA_A$ receptors was clearly more reduced when brain sections were incubated with 2 μM GABA (Fig. 4B). The results are in agreement with our findings that GAA acts as an agonist on neuronal $GABA_A$ receptors in cortex and GP. The experiment also indicated weaker agonistic properties of GAA as compared to GABA.

GAA Acts as an Agonist on Recombinant $GABA_A$ Receptors, But Not on Recombinant $GABA_B$ Receptors

To assess whether the GAA effects observed in our electrophysiological and radioligand binding experiments are due to a direct GABA-mimetic action of GAA on $GABA_A$ receptors, we expressed the main $GABA_A$ receptor subtype $\alpha1\beta2\gamma2$ in *Xenopus* oocytes. These subunits are also present in GP (Lüddens *et al.*, 1990). The application of GAA at concentrations from 100 μM to 2 mM evoked dose-dependent inward currents that deactivated after GAA washout (Fig. 6A), confirming that GAA acts as a direct $GABA_A$ agonist. Application of a saturating GABA concentration (2 mM) induced currents about five times larger than 2 mM GAA (Fig. 6A, right current trace). This is reflected in the normalized concentration–response relationship for GABA and GAA (Fig. 6B). The respective fits of the Hill function provided EC_{50} values of 40 ± 4 μM and 1.30 ± 0.13 mM for GABA and GAA, respectively. The Hill coefficients were similar for both agonists (GABA: 1.21 ± 0.17; GAA: 1.09 ± 0.06). The extrapolated maximum of the fit to the GAA data points was 0.31 ± 0.01 mM, illustrating a weaker

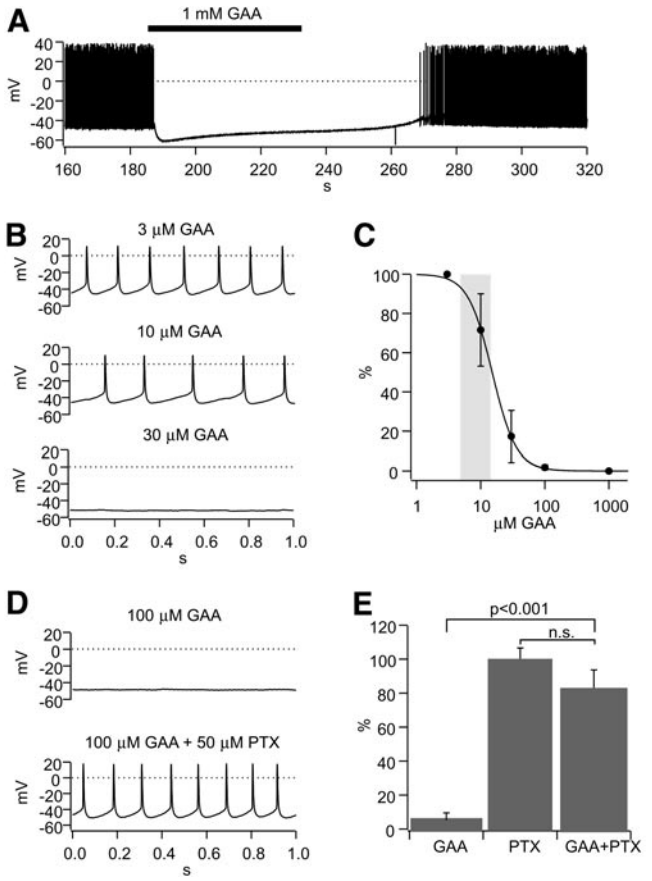

FIG. 4. GAA inhibits sustained neuronal activity at relevant concentrations. Current-clamp recordings of fast-spiking central neurons in acute brain slices containing the globus pallidus by the use of a low internal chloride concentration (4 mM). (A) Application of GAA (1 mM) led to hyperpolarization of the GP neuron and inhibition of pacemaker activity. This effect was reversible after washout. (B) Current-clamp recordings of a fast-spiking neuron, displaying pacemaker activity under various GAA concentrations. GAA (3 μM) did not affect the spike frequency, whereas 10 or 30 μM, respectively, inhibited spiking to 71.7 $\pm$ 18.4% (n = 5) and 17.4 $\pm$ 13.2% (n = 7) of control conditions. (C) The relative change in spiking activity was dose dependent and was fit by a Hill function with an EC_{50} of 15.1 μM and a Hill coefficient of 2.3. The concentration range found in the CSF of GAMT patients is shown in gray. (D) Application of GAA (100 μM) under control conditions inhibited neuronal activity (upper trace). In the presence of PTX (50 μM), the effect of GAA (100 μM) on the same cell was largely masked (bottom trace). (E) Statistical analysis of the results obtained in (D) expressed in percent of spike frequencies recorded in the presence of 50 μM PTX. Application of 100 μM GAA reduced spike frequency to 4.9 $\pm$ 4.7%, whereas coapplication of 100 μM GAA and 50 μM PTX did not induce significant changes (84.4 $\pm$ 9.3%).

agonistic activity of GAA on this $GABA_A$ receptor subtype. Next, we examined whether GAA, due to its smaller maximum efficacy, might be capable of inhibiting GABA responses. We first stimulated oocytes with 0.3 mM GABA or 5 mM GAA. At these concentrations, the respective agonist should occupy 80–90% of the receptors. Coapplication of both agonists did not have an additive effect, but evoked responses of intermediate amplitude (Fig. 6C).

Since GABA and GAA are structurally very similar and GAA shows GABA-mimetic properties on native and cloned $GABA_A$ receptors, we examined the GABA receptor specificity of GAA binding. We therefore transiently transfected a stable $GABA_B$ ($GABA_BR1a$/$GABA_BR2$) receptor CHO cell line with G protein-regulated Kir3.1/Kir3.2 (GIRK) potassium channels and analyzed currents resulting from receptor activation by using a ramp voltage-clamp protocol from −120 to 0 mV. Application of GAA at concentrations of up to 1 mM did not result in detectable currents mediated by Kir3.1/Kir3.2 (Fig. 7A), indicating that GAA did not act as agonist on $GABA_B$ receptors. We subsequently tested whether GAA might act as antagonist on $GABA_B$ receptors and coapplied GABA and GAA in order to analyze the resulting GIRK-mediated current amplitudes. As opposed to recombinant $GABA_A$ receptors, GAA did not change GABA-induced currents (Figs. 7B and 7C). Thus, GAA acts neither as agonist nor as antagonist at $GABA_B$ receptors.

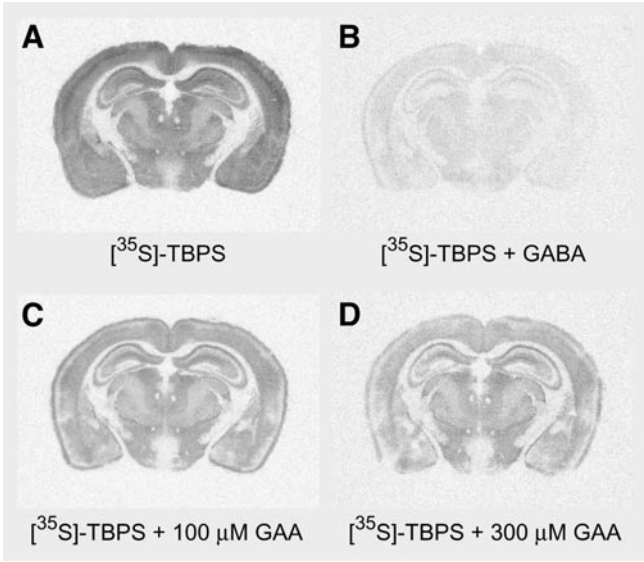

FIG. 5. Reduction of [^{35}S]TBPS binding through GAA. Representative autoradiographs of [^{35}S]TBPS binding under basal conditions (A) and in the presence of 2 μM GABA (B) and 100 μM (C) or 300 μM GAA (D). The images demonstrate the decrease in [^{35}S]TBPS binding through coincubation with $GABA_A$ receptor agonists GABA and GAA.

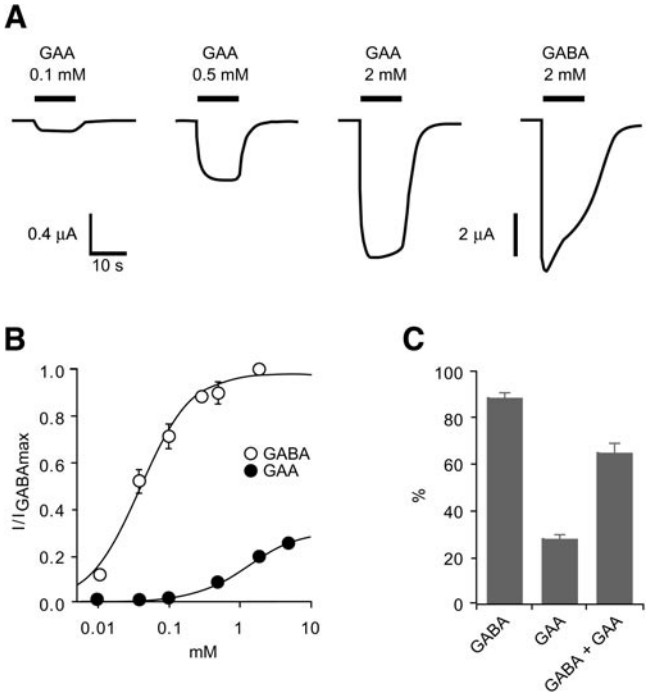

FIG. 6. GAA activates cloned $GABA_A$ receptors expressed in *Xenopus* oocytes. (A) Examples of current responses evoked by the application of GAA (0.1, 0.5, and 2 mM) or GABA (2 mM, recorded at fivefold lower gain). For the analysis shown in (B) and (C), current amplitudes were measured 10 s after the start of each agonist application. (B) Concentration–response relations for GABA (open circles) and GAA (filled circles). Current amplitudes were expressed as a fraction of the current evoked by 2 mM GABA (three to seven oocytes per data point; error bars not shown if SEM was smaller than the symbol size). The fits of the Hill function (curves) provided EC_{50} values of 0.040 ± 0.004 and 1.30 ± 0.13 mM for GABA and GAA, respectively. The Hill coefficients were similar for both agonists (1.21 ± 0.17 and 1.09 ± 0.06). The extrapolated maximum of the fit to the GAA data was 0.31 ± 0.01. (C) GAA partially inhibits GABA responses. Three oocytes were first stimulated by GABA (0.3 mM, left bar) and GAA (5 mM, middle bar). At these concentrations, the agonists should occupy 80–90% of the receptors. Coapplication of both agonists did not have an additive effect, but evoked responses of intermediate amplitude (right bar). Current amplitudes were normalized as in (B) (GABA 2 mM as 100%).

Collectively, our experiments confirmed that GAA directly interacts with native and recombinant $GABA_A$ receptors and thus activates the $GABA_A$ chloride channels in a dose-dependent manner.

DISCUSSION

Guanidinoacetate Interacts with $GABA_A$ Receptors

GAA is the only metabolite with a dramatically increased concentration in CSF, serum, and urine of patients with GAMT deficiency. Our electrophysiological and pharmacological results demonstrate that clinically relevant concentrations of GAA activate neuronal $GABA_A$ receptor channels. Fast application experiments suggest that GAA acts as a direct agonist on $GABA_A$ receptors with micromolar affinities. This finding was supported by the GAA-induced activation of $GABA_A$ receptors expressed in *Xenopus* oocytes. The latter experiments also indicated that GAA has a weaker GABA-mimetic potential than GABA that is also reflected in the smaller inhibition of [^{35}S]TBPS binding visible in the functional [^{35}S]TBPS ligand autoradiography experiments. Since coapplication of GABA and GAA on recombinant $GABA_A$ receptors yielded currents of intermediate amplitude, GAA may also exhibit antagonistic properties *in vivo*. The final pathophysiological effect on target neurons like those in the globus pallidus may therefore critically depend on the local GABA concentration. The uremic guanidino compounds creatinine, guanidine, guanidinosuccinic acid, and methylguanidine are known to have an epileptogenic potential, both *in vitro* and *in vivo* (D'Hooge *et al.*, 1992; De Deyn and Macdonald, 1990; De Deyn *et al.*, 1991), and were shown to block GABA responses on spinal cord neurons at millimolar concentrations (D'Hooge *et al.*, 1999). In contrast, even micromolar GAA concentrations activated

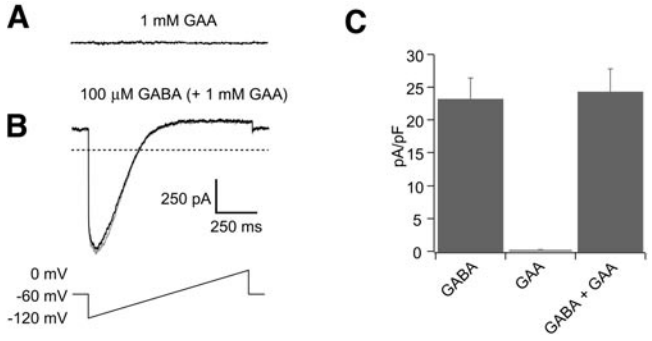

FIG. 7. No effect of GAA on $GABA_B$ receptors. Voltage-clamp recordings of CHO cells stably expressing $GABA_B$ receptor subunits $GABA_B$R1a/$GABA_B$R2. To measure receptor activation, cells were transiently transfected with Kir3.1/Kir3.2 subunits. Currents were recorded by a ramp protocol ranging from −120 to 0 mV. Net currents were obtained by subtracting control recordings from recordings after application of substances. (A) GAA (1 mM) did not activate $GABA_B$ receptors. (B) Application of GABA (100 μM) elicited inward-rectifying currents, demonstrating functional expression of $GABA_B$ receptors and Kir3.1/Kir3.2 channels (black trace). The GABA response was not altered by coapplication of GAA (1 mM) (gray trace). (C) Current densities obtained from experiments in (A) and (B).

$GABA_A$ receptors in our experiments. The physiological GAA concentration in human CSF and plasma is in the nanomolar range, which is clearly not sufficient to activate $GABA_A$ receptors. In GAMT deficiency, however, concentrations between 10 and 30 μM were found (Leuzzi *et al.*, 2000; Schulze *et al.*, 1998; Stöckler *et al.*, 1997). These clinically relevant GAA concentrations substantially impaired spontaneous activity of cultured cortical neurons as well as that of pallidal neurons in *in vitro* brain slices. The increase in GAA concentrations in GAMT deficiency may lead to relevant changes in $GABA_A$ receptor activity in different ways. Upon prolonged (chronic) stimulation, $GABA_A$ receptors undergo desensitization. The resulting decrease in channel availability and the smaller GABA responses would, in turn, increase neuronal excitability. Depending on the local GABA concentration, GAA might also reduce maximal $GABA_A$ receptor activation by directly antagonizing GABA responses. Another possibility of an excitatory action induced by GAA might be an activity-dependent, depolarizing shift of the reversal potential of the permeant ion chloride due to prolonged activation of $GABA_A$ receptors (Staley and Proctor, 1999; Staley *et al.*, 1995).

The basal ganglia are mainly interconnected by GABAergic projections and play an important role in motor activity. The basal ganglia output nuclei globus pallidus and substantia nigra pars reticulata are involved in both movement and seizure control (Depaulis *et al.*, 1994). In our experiments, GAA changed the spike pattern of globus pallidus neurons in *in vitro* brain slices via $GABA_A$ receptors. Motor symptoms as well as epileptic seizures in patients with GAMT deficiency could therefore be explained by interference of GAA with the inhibitory GABAergic circuits of the basal ganglia.

Therapeutic Implications

Anticonvulsant therapy used in GAMT patients with seizures was ineffective (Leuzzi *et al.*, 2000; Schulze *et al.*, 1997; Stöckler *et al.*, 1996b). The current therapeutic approach consists of oral creatine supplementation, which results in limited improvement of the clinical symptoms (Ganesan *et al.*, 1997; Leuzzi *et al.*, 2000; Stöckler *et al.*, 1996a). However, creatine supplementation alone is not sufficient to completely normalize clinical and/or biochemical findings. In particular, GAA concentrations in plasma and urine of creatine-treated GAMT patients remain significantly elevated, indicating that even high-normal creatine concentrations do not completely inhibit GAA formation by arginine:glycine amidinotransferase. Our findings suggest that GAA might be an active key metabolite causing neurological disease in GAMT deficiency and thus provide an explanation why the use of drugs potentiating GABAergic neurotransmission is ineffective. In addition, our results give a clear rationale for avoiding the use of these drugs in GAMT deficiency. Instead, therapeutic approaches decreasing intracerebral GAA concentrations are needed to successfully treat GAMT patients. Recently, a dietary therapeutic approach consisting of a combination of creatine supplementation, arginine restriction, and administration of ornithine resulted in decreased plasma, CSF, and urine GAA concentrations and improved the clinical condition of a patient by reducing seizure activity (Schulze *et al.*, 2001). Before this treatment, the patient had not responded to a therapeutic trial consisting of creatine substitution and arginine restriction (Schulze *et al.*, 1998), since it had not changed GAA levels substantially. These clinical findings underscore the importance of GAA in the pathophysiology of GAMT deficiency.

In summary, we provide experimental evidence that the naturally occurring guanidino compound GAA that accumulates in a metabolic human disease acts as a partial $GABA_A$ receptor agonist. We thus propose a novel mechanism that might be active *in vivo* and hence could contribute to the neurological disturbances observed in GAMT deficiency.

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