Pretreatment of Guinea Pigs with Galantamine Prevents Immediate and Delayed Effects of Soman on Inhibitory Synaptic Transmission in the Hippocampus

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ABSTRACT

Galantamine has emerged as a potential antidote to prevent the acute toxicity of organophosphorus (OP) compounds. Changes in inhibitory GABAergic activity in different brain regions can contribute to both induction and maintenance of seizures in subjects exposed to the OP nerve agent soman. Here, we tested the hypothesis that galantamine can prevent immediate and delayed effects of soman on hippocampal inhibitory synaptic transmission. Spontaneous inhibitory postsynaptic currents (IPSCs) were recorded from CA1 pyramidal neurons in hippocampal slices obtained at 1 h, 24 h, or 6 to 9 days after the injection of guinea pigs with saline (0.5 ml/kg i.m.), 1×LD₅₀ soman (26.3 μg/kg s.c.), galantamine (8 mg/kg i.m.), or galantamine at 30 min before soman. Soman-challenged animals that were not pretreated showed mild, moderate, or severe

signs of acute intoxication. At 1 h after the soman injection, the mean IPSC amplitude recorded from slices of mildly intoxicated animals and the mean IPSC frequency recorded from slices of severely intoxicated animals were larger and lower, respectively, than those recorded from slices of control animals. Regardless of the severity of the acute toxicity, at 24 h after the soman challenge the mean IPSC frequency was lower than that recorded from slices of control animals. At 6 to 9 days after the challenge, the IPSC frequency had returned to control levels, whereas the mean IPSC amplitude became larger than control. Pretreatment with galantamine prevented soman-induced changes in IPSCs. Counteracting the effects of soman on inhibitory transmission can be an important determinant of the antidotal effectiveness of galantamine.

Introduction

Organophosphorus (OP) nerve agents, including soman, sarin, and VX, are among the most lethal chemical warfare agents. They are chemically related to, although far more toxic than, OP pesticides used in agriculture and households worldwide. Although OPs interact with numerous molecular targets (Albuquerque et al., 1985; Huff et al., 1994; Duysen et al., 2001), acute OP intoxication results primarily from the irreversible inhibition of acetylcholinesterase (AChE) that leads to acetylcholine (ACh) accumulation and, consequently,

overstimulation of cholinergic receptors in the peripheral and central nervous systems (Newmark, 2007).

Changes in the activity of the excitatory glutamatergic and the inhibitory GABAergic systems in brain regions enriched with cholinergic inputs, including the hippocampus, seem to contribute to the maintenance of OP-induced seizures (Shih and McDonough, 1997; Myhrer, 2007). In hippocampal microdialysates, levels of the inhibitory neurotransmitter GABA have been shown to decrease in guinea pigs at 1 to 2 h after exposure to soman (Fosbraey et al., 1990). On the other hand, levels of GABA have been found to be significantly increased in hippocampal tissue obtained from rats at 80 min after the onset of soman-induced seizures (Shih and McDonough, 1997). Neurotransmitter levels detected in microdialysates reflect both synaptic release and nonspecific overflow from synaptic and nonsynaptic (metabolic) sources. Likewise, tissue levels of neurotransmitters reflect total (metabolic and synaptic) contents of the transmitter. Thus, very little is known regarding the immediate and protracted effects of an acute in vivo exposure to soman on GABAergic synaptic transmission in the hippocampus.

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ABBREVIATIONS: OP, organophosphorus; ACh, acetylcholine; AChE, acetylcholinesterase; IPSC, inhibitory postsynaptic current; IACUC, Institutional Animal Care and Use Committee; ANOVA, analysis of variance; nAChR, nicotinic acetylcholine receptor; mAChR, muscarinic acetylcholine receptor; ACSF, artificial cerebrospinal fluid.

Galantamine prevents the acute toxicity of OP nerve agents and pesticides in guinea pigs, the best nonprimate model to predict the effectiveness of OP antidotes in humans (Albuquerque et al., 2005, 2006; Pereira et al., 2008). Galantamine, a drug approved for treatment of mild to moderate Alzheimer's disease, has a dual mode of action; it is a reversible, competitive AChE inhibitor and a positive allosteric modulator of nAChRs (Maelicke and Albuquerque, 2000; Pereira et al., 2002). However, the actions of galantamine that contribute to its effectiveness as a therapeutic countermeasure against OP poisoning remain poorly understood.

The present study was designed to test the hypothesis that an acute exposure of guinea pigs to soman has immediate and delayed effects on GABAergic transmission in the CA1 field of the hippocampus that are preventable by pretreatment of the animals with galantamine. To test this hypothesis, the whole-cell patch-clamp technique was used to record spontaneous inhibitory postsynaptic currents (IPSCs) from CA1 pyramidal neurons in hippocampal slices obtained at 1 h, 24 h, and 6 to 9 days after a single exposure guinea pigs to of soman and/or galantamine.

Evidence is provided herein that GABAergic transmission impinging onto CA1 pyramidal neurons changes significantly with time after a subcutaneous injection of guinea pigs with soman ($1 \times \text{LD}_{50}$). At 1 h after the exposure, an increase in the IPSC amplitudes was observed in the hippocampi of mildly intoxicated animals, and a decrease in the frequency of IPSCs was seen in the hippocampi of severely intoxicated animals. Regardless of the severity of the acute toxicity exhibited by the animals, the IPSC frequency was lower and the IPSC amplitudes were larger than control at 24 h and 6 to 9 days after the exposure, respectively. Although a single intramuscular injection of galantamine (8 mg/kg) had no long-term effects on the IPSCs in the CA1 region, pretreatment of the guinea pigs with galantamine prevented the effects of soman. Galantamine's ability to prevent the effects of soman on GABAergic transmission may contribute to its effectiveness as an antidotal therapy against soman poisoning.

Materials and Methods

Animal Care and Treatments. Female Hartley guinea pigs [CrI(HA)Br; Charles River Laboratories, Inc., Wilmington, MA] were housed in groups of four in stainless-steel cages ($60 \times 60 \times 25$ cm) in a climate-controlled animal care facility with constant temperature of $21 \pm 0.5^\circ\text{C}$ and a 12-h light/dark cycle. Animals were 30 to 33 days old upon arrival and were acclimated for at least 48 h before any treatment. The study complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Guinea pigs were divided into four treatment groups. Group I ($n = 30$) received an injection of saline (0.9% NaCl; 0.5 ml/kg i.m.). Group II ($n = 49$) received an injection of $26.3 \mu\text{g/kg}$ soman ($1 \times \text{LD}_{50}$ s.c.). Group III ($n = 19$) was treated with galantamine (8 mg/kg i.m.) 30 min before the challenge with $26.3 \mu\text{g/kg}$ s.c. soman. This treatment regimen was selected on the basis of our earlier demonstration that all guinea pigs survive with no signs of acute toxicity when they are treated with galantamine (8 mg/kg i.m.) and 30 min later exposed to $1 \times \text{LD}_{50}$ soman (Aracava et al., 2009; Gullapalli et al., 2010). Group IV ($n = 30$) received only galantamine (8 mg/kg i.m.).

Experiments were conducted at 1 h, 24 h, and 6 to 9 days after the injections. Animals were monitored every 15 min during the first 2 h

after the injections, hourly during the next 6 h, and daily subsequently. Acute reactions to treatments were scored according to a modified Racine scale (Aracava et al., 2009) as described under *Results*. The experimenter who performed the electrophysiological experiments and analyzed the data was blind to the severity of the acute toxicity. Whenever animals developed life-threatening signs of intoxication, including gasping and unremitting motor convulsions, they were euthanized according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Preparation of Hippocampal Slices. Following the IACUC guidelines, guinea pigs were euthanized by asphyxiation in a CO_2 atmosphere followed by decapitation. Their brains were removed, and the hippocampi were dissected out in cold artificial cerebrospinal fluid (ACSF). The middle third section of each hippocampus was used for slicing. Using a vibratome (Leica VT1000S; Leica Microsystems Inc., Deerfield, IL), transverse slices (300- μm thick) were cut in ice-cold ACSF bubbled with 95% O_2 /5% CO_2 . They were maintained for at least 1 h at room temperature in a humidified environment on the surface of ACSF that was continuously bubbled with 95% O_2 /5% CO_2 . ACSF was composed of 125 mM NaCl, 25 mM NaHCO_3 , 2.5 mM KCl, 2 mM CaCl_2 , 1.6 mM MgCl_2 , and 11 mM D-glucose.

Electrophysiological Recordings. Recordings were obtained at room temperature (20 – 22°C) from the soma of CA1 pyramidal neurons by using the conventional whole-cell patch-clamp technique and an LM-EPC7 amplifier (List Electronics, Darmstadt, Germany). Hippocampal slices were transferred to the recording chamber and perfused with oxygenated ACSF at 2 ml/min. In the chamber, they were kept submerged in ACSF and held in place by two nylon fibers. Patch pipettes were pulled from borosilicate glass capillaries (i.d. 1.2 mm; World Precision Instruments Inc., Sarasota, FL) with a P-97 Flaming-Brown puller (Sutter Instrument Company, Novato, CA) and had resistances of 3.5 to 6.5 M Ω when filled with the internal solution. The internal solution was composed of 130 mM Cs-methane sulfonate, 10 mM CsCl, 2 mM MgCl_2 , 10 mM EGTA, 10 mM HEPES, and 5 mM lidocaine *N*-ethyl bromide. The pH was adjusted to 7.3 with CsOH. Biocytin (final concentration 0.3–0.5%) was added to the pipette solution to label the neurons from which recordings were obtained.

Neurons were visualized by infrared-assisted video microscopy. Currents were filtered at 3 kHz, digitized at 10 kHz, and recorded with pClamp9.2 software (Molecular Devices, Sunnyvale, CA). The series resistance estimated from the amplitude of the initial capacitive transient in response to a 5-mV pulse was 8 to 24 M Ω . It was not compensated and was monitored during each experiment. Experiments were terminated if the series resistance changed by more than 15%. Spontaneous IPSCs were recorded for 5 min at the reversal potential for glutamatergic currents (i.e., 0 mV). The GABA_A receptor antagonist bicuculline (10 μM) was applied at the end of the experiments to confirm that the synaptic events were indeed GABAergic in nature.

Data Analysis and Statistics. The Mini Analysis 6.0.3 software (Synaptosoft Inc., Decatur, GA) was used to analyze the frequency and amplitude of synaptic events. Amplitudes, rise times (10–90%), decay-time constants (τ_d), and area (pA $\times$ ms) of single events were also measured as described below. The threshold amplitude for detecting IPSCs was set at twice the baseline noise (root mean square), and the IPSCs detected by the software were visually inspected to minimize errors. Events that did not show a typical synaptic wave form were rejected manually.

For kinetic analysis, only single events with a sharp rising phase and an exponential decay were chosen during visual inspection of the recordings. Double- and multiple-peak currents were excluded. Rise times and τ_d were determined during the analysis of the averaged chosen single events aligned at half rise time in each cell. More than 90% of the synaptic events were fitted with a single exponential decay. Charge transfer of IPSCs was taken as the area under the curve of the currents. To minimize potential sampling bias, a maximum of two cells per animal was studied. Data are expressed as

mean $\pm$ S.E.M. of results obtained from various animals, and statistical significance was analyzed by using ANOVA or the Mann-Whitney test.

Drugs. Stock solution of soman (1.88–1.9 mg/ml) was obtained from the U.S. Army Edgewood Chemical Biological Center via an agreement with the U.S. Army Medical Research Institute of Chemical Defense. Soman (methylphosphonofluoridic acid 1,2,2-trimethylpropyl ester) was stored, handled, and disposed of according to the regulations set forth by the U.S. Army Medical Research Institute of Chemical Defense. Galantamine-HBr was generously provided by Dr. Alfred Maelicke (Galantos Pharma, Mainz, Germany).

Results

Acute Effects and Lethality of $1 \times LD_{50}$ Soman Are Prevented by Treatment of the Guinea Pigs with Galantamine. The severity of the acute toxicity presented by guinea pigs challenged with $1 \times LD_{50}$ soman was qualitatively defined according to a modified Racine scale as described in Aracava et al. (2009). Animals were considered mildly (stages 0 and 1), moderately (stages 2 and 3), and severely intoxicated (stages 4 and 5) (see Table 1). In animals that reached stages 1 to 3, signs of acute intoxication were not life-threatening and subsided within a few hours after the soman challenge. According to the IACUC-approved protocol, animals had to be euthanized as soon as signs of intoxication became life-threatening. Thus, only a few animals that reached stages 4 and 5 remained alive after 24 h of exposure to soman.

Of 90 soman-injected guinea pigs, 49 were used in the present electrophysiological studies (see Table 1). Of those, 38 animals did not go beyond stage 3; 8, 13, and 17 animals were euthanized at 1 h, 24 h and 6 to 9 days after the challenge, respectively. Seven of the other 11 animals reached stage 4 within 45 to 60 min and were euthanized at 1 h after challenge. The remaining four reached stage 4 within a few hours. Two of these animals were euthanized and used at 24 h, because they still displayed signs of toxicity at that time. The other two guinea pigs were euthanized and used between days 6 and 9 after the challenge. As soon as the animals were euthanized, their brains were removed and their hippocampi were dissected for slicing as described under *Materials and Methods*.

At 24 h after the injection of soman, the surviving guinea pigs had lost 10 to 15% of their initial weight. At 6 to 9 days, they were gaining weight, albeit at a rate slower than control (saline-injected) animals, and could not be distinguished from controls on the basis of their gross behavior.

As in previous studies (Alkondon et al., 2009; Aracava et al., 2009; Gullapalli et al., 2010), guinea pigs treated with galantamine (8 mg/kg i.m.) alone or challenged with soman 30 min after the treatment with galantamine survived with no apparent signs of acute toxicity and gained weight at the same rate as control animals (data not shown).

Characteristics of IPSCs Recorded from CA1 Pyramidal Neurons in Guinea Pig Hippocampal Slices. Spontaneous postsynaptic currents recorded from CA1 pyramidal neurons at the reversal potential of glutamatergic events (0 mV) were outward events. These events were blocked by the GABA_A receptor antagonist bicuculline (10 μ M) (Fig. 1A). In addition, in the presence of the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (10 μ M) and 2-amino-5-phosphonopentanoic acid (50 μ M), the synaptic currents reversed between –30 and –60 mV (Fig. 1B). Based on the Nernst equation, the null potential for Cl[–] under the present experimental condition is approximately –52 mV. Thus, outward currents recorded at 0 mV are primarily IPSCs mediated by GABA_A receptors.

The frequency (expressed as number of IPSCs/recording time), amplitude, τ_d , and rise time of IPSCs recorded from slices obtained at 1 h were not significantly different from those recorded at 24 h or 6 to 9 days after the saline injection (Table 2). Therefore, results from all control animals were pooled together for subsequent comparison with results obtained from soman-exposed animals.

Immediate and Delayed Effects of the Acute Exposure of Guinea Pigs to Soman on IPSCs. The mean frequency and amplitude of IPSCs recorded from hippocampal slices taken from guinea pigs at 24 h or 6 to 9 days after soman challenge did not vary with the animals' score of acute toxicity (Fig. 2A). Therefore, for subsequent comparison with control values, data from all animals were pooled together for each time point. Data obtained at 1 h after soman could not be pooled, because the mean IPSC amplitudes were significantly higher in mildly intoxicated (scores 0–1) than in moderately intoxicated (scores 2–3) or severely intoxicated (scores 4–5) animals (Fig. 2B).

At 1 h after soman, the IPSC amplitudes recorded from slices of mildly intoxicated guinea pigs were significantly larger than control (Fig. 3A). In those slices, the IPSC frequency remained unchanged (Fig. 3B). On the other hand, in slices obtained at 1 h after soman from severely intoxicated animals the IPSC frequency was significantly lower than

TABLE 1

Classification of the severity of soman-induced toxicity

The severity of the acute toxicity presented by guinea pigs challenged with $1 \times LD_{50}$ soman was classified as described in Aracava et al. (2009). According to the IACUC-approved protocol, animals had to be euthanized whenever signs of intoxication became life threatening. The far-right column shows the number of animals that were not used in this electrophysiological study because they had to be euthanized earlier than the experimental time points.

Stage	Progression of Behavioral Changes	Classification of Toxicity	Number of Animals			
			Euthanized and Used in Experiments			Euthanized, Not Used
			1 h	24 h	6–9 Days	
0	No abnormal gross behavior	Mild	3	8	11	0
1	Facial twitches, pawing at whiskers and mouth, chewing	Mild	3	8	11	0
2	Head tremor and/or nodding, short periods of immobility	Moderate	5	5	6	2
3	Forelimb clonus	Moderate	5	5	6	2
4	Rearing with no loss of balance, strong grinding, gnashing or bruxism	Severe	7	2	2	39
5	Rearing with loss of balance, frank convulsions	Severe	7	2	2	39

Fig. 1. Sample recordings of spontaneous IPSCs obtained from CA1 pyramidal neurons in hippocampal slices obtained from saline-injected guinea pigs. A, postsynaptic currents recorded from a CA1 pyramidal neuron at 0 mV before (control) and 10 min after perfusion of the slice with ACSF containing the GABA_A receptor antagonist bicuculline (10 μ M). B, representative traces of IPSCs recorded from another neuron at different membrane potentials. ACSF contained the glutamate receptor antagonists 2-amino-5-phosphonopentanoic acid (50 μ M) and 6-cyano-7-nitroquinoxaline-2,3-dione (10 μ M).

TABLE 2

Characteristics of IPSCs recorded from CA1 pyramidal neurons in hippocampal slices taken from guinea pigs at various times after a single injection of saline

Data are presented as mean $\pm$ S.E.M. of results obtained from 10 neurons (1 neuron/animal) for each time point.

	Time after Injection of Saline		
	1 h	24 h	6–9 Days
Frequency (Hz)	0.87 $\pm$ 0.10	0.94 $\pm$ 0.16	0.89 $\pm$ 0.10
Amplitude (pA)	15.1 $\pm$ 0.8	15.7 $\pm$ 1.0	15.6 $\pm$ 0.6
τ_d (ms)	24.2 $\pm$ 1.6	24.9 $\pm$ 1.9	25.6 $\pm$ 1.7
Rise time (10–90%, ms)	1.85 $\pm$ 0.2	1.84 $\pm$ 0.29	2.04 $\pm$ 0.34
Net Charge (pA·ms)	400 $\pm$ 38	429 $\pm$ 45	403 $\pm$ 40

control (Fig. 3B); there was no concomitant change in the IPSC amplitude (Fig. 3A).

At 24 h after soman challenge, the frequency of IPSCs was significantly lower than control (Fig. 4A). The cumulative distribution of interevent intervals was also shifted toward longer intervals in comparison with control (Fig. 4B). The reduction of IPSC frequency was transient, because it was no longer detected in slices taken at 6 to 9 days after soman challenge (Fig. 4A).

The mean amplitude of IPSCs recorded at 24 h after soman was comparable with control. However, at 6 to 9 days after soman, it was significantly larger than control (Fig. 4C); the histogram and cumulative distribution of IPSC amplitudes were also skewed toward larger ampli-

Fig. 2. Relationship between the acute toxicity and the mean IPSC frequency and amplitude recorded from hippocampal slices taken at different times from guinea pigs exposed to $1 \times LD_{50}$ soman. Box plots of toxicity scores versus frequency (A) or amplitude (B) of IPSCs in slices obtained at 1 h, 24 h, or 6 to 9 days after the injection of guinea pigs with soman (26.3 μ g/kg s.c.). Scores refer to mild (0–1), moderate (2–3), and severe (4–5) signs of acute intoxication, as described in Table 1. Numbers of animals per group are shown in parentheses. The IPSC amplitudes recorded from CA1 pyramidal neurons in slices taken at 1 h after soman challenge decreased as the severity of intoxication increased. *, $p < 0.05$ and ***, $p < 0.001$ according to ANOVA followed by Bonferroni post hoc test.

Fig. 3. Changes in frequency and amplitude of IPSCs recorded from CA1 pyramidal neurons in slices taken at 1 h after soman ($1 \times LD_{50}$) challenge. A, amplitudes of IPSCs recorded from neurons in slices from mildly intoxicated guinea pigs (scores 0–1) were significantly higher than those recorded from neurons in slices of saline-injected (control) animals. B, graph shows the frequency of IPSCs recorded from pyramidal neurons from the same slices as in A. IPSC frequency in slices from severely intoxicated guinea pigs (scores 4–5) was significantly lower than control. Graph and error bars represent mean and S.E.M., respectively, obtained from the following number of neurons: 30 (saline), 3 (scores 0–1), 9 (scores 2–3), and 7 (scores 4–5). *, $p < 0.05$ according to ANOVA followed by Dunnett post hoc comparison with control.

tudes (Fig. 4D). The charge transfer through individual IPSCs recorded from control animals was 410 ± 41 pA·ms and increased to 585 ± 74 pA·ms at 6 to 9 days after soman challenge ($p < 0.05$ according to the unpaired Student's t test). This increase was caused by the increase in IPSC amplitudes, because the exposure to soman had no significant effect on the τ_d of the events. At 1 h, 24 h, and 6 to 9 days after soman, τ_d s of the IPSCs were 26.3 ± 1.5 , 26.1 ± 1.3 , and 24.3 ± 0.7 ms, and their rise times were 2.05 ± 0.33 , 1.82 ± 0.13 , and 1.88 ± 0.15 ms, respectively. These results are comparable with those obtained from control animals (see Table 1).

Effects of an Acute Treatment with Galantamine on IPSCs Impinging onto CA1 Pyramidal Neurons in Naive and Soman-Injected Guinea Pigs. Pretreatment with galantamine (8 mg/kg i.m.) prevented the effects of soman

Fig. 4. Changes in IPSC frequency and amplitude recorded from CA1 pyramidal neurons at 24 h or 6 to 9 days after injection of guinea pigs with $1 \times LD_{50}$ soman. A, mean frequency of IPSCs recorded from neurons in slices taken at 24 h after soman was significantly lower than control. **, $p < 0.01$ according to ANOVA followed by Dunnett post hoc comparison with control. B, cumulative probability plot of inter-event intervals recorded at 24 h after soman was displaced to the right in comparison with control. $p < 0.05$ according to the Mann-Whitney test. C, mean amplitudes of IPSCs recorded from neurons in slices obtained at 6 to 9 days after soman were significantly larger than control. *, $p < 0.05$ according to ANOVA followed by Dunnett post hoc comparison with control. D, compared with control, the histogram and cumulative probability plot of IPSC amplitudes recorded at 6 to 9 days after soman were skewed toward larger amplitudes. $p < 0.05$ according to the Mann-Whitney test. Data are presented as mean and S.E.M. of results obtained from 30 (saline), 21 (24 h after soman), and 22 neurons (6–9 days after soman).

on the frequency and amplitudes of IPSCs (Fig. 5). In slices from galantamine-treated, soman-challenged guinea pigs, the frequency and amplitude of IPSCs were comparable with control (Fig. 5).

In guinea pigs treated intramuscularly with 8 mg/kg galantamine, brain concentrations of the drug peak at 30 min after the injection and decay with a half-life of approximately 70 min (Albuquerque et al., 2006). Pharmacologically relevant concentrations of galantamine remain present in brain

Fig. 5. Pretreatment of the guinea pigs with galantamine prevented soman-induced changes in the frequency and amplitude of IPSCs. Animals were treated with galantamine (8 mg/kg i.m.) and 30 min later they were challenged with $1 \times LD_{50}$ soman (26.3 μ g/kg s.c.). Hippocampal slices were obtained from animals at 1 h, 24 h, or 6 to 9 days after the soman challenge. Graphs show frequency (A) and amplitudes (B) of IPSCs in galantamine-treated, soman-challenged animals were comparable with control levels. Graph and error bars represent mean and S.E.M., respectively, of results obtained from the following number of neurons: 30 (saline), 12 (1 h after soman), 13 (24 h after soman), and 10 (6–9 days after soman).

tissue up to 3 h after treatment. Thus, during the first 3 h after the injection of galantamine, timing to obtain the hippocampal slices is an important determinant of the remaining tissue concentrations of the drug. For meaningful comparison of results, hippocampal slices were obtained at 1.5 h after treatment with galantamine alone, because they were also obtained at 1.5 h after the galantamine treatment of soman-challenged animals.

At 1.5 h, 24 h, or 6 to 9 days after the injection of galantamine, the frequency of IPSCs was not significantly different from control (Fig. 6A). However, at 1.5 h after galantamine the IPSC amplitudes were larger than control (Fig. 6B); the histogram distribution and cumulative plot of IPSC amplitudes were also shifted toward larger amplitudes (Fig. 6C). The effect of galantamine on the IPSC amplitudes was transient and could not be detected at 24 h or 6 to 9 days after the treatment (Fig. 6B).

Fig. 6. Effects of the acute treatment of guinea pigs with galantamine on IPSC frequency and amplitudes. A and B, graphs of mean IPSC frequency (A) and amplitudes (B) recorded from CA1 pyramidal neurons in slices obtained at 1.5 h, 24 h, or 6 to 9 days after the injection of guinea pigs with galantamine (8 mg/kg i.m.). Treatment with galantamine caused a significant transient increase in the IPSC amplitudes. *, $p < 0.05$ according to ANOVA followed by Dunnett post hoc comparison with control. C, the histogram and cumulative probability plot of IPSC amplitudes recorded at 1.5 h after the injection of galantamine were displaced toward larger amplitudes in comparison with control. $p < 0.05$ according to the Mann-Whitney test. Data are presented as mean and S.E.M. of results obtained from the following number of neurons: 30 (saline), 17 (galantamine, 1.5 h), 12 (galantamine, 24 h), and 11 (galantamine, 6–9 days).

The rise time of IPSCs recorded at 1.5 h, 24 h, and 6 to 9 days after the galantamine treatment were 1.97 ± 0.29 , 2.17 ± 0.46 , and 1.78 ± 0.23 ms, respectively. The τ s of these events were 22.9 ± 1.3 , 22.3 ± 1.9 , and 25.4 ± 1.8 ms, respectively. These parameters were not significantly different from control (see Table 1).

Discussion

The present study demonstrates that an acute exposure of guinea pigs to soman has distinct immediate and delayed effects on GABAergic synaptic transmission impinging onto CA1 pyramidal neurons. The ability of galantamine to prevent these effects can be an important determinant of its antidotal effectiveness.

Immediate and Delayed Effects of an Acute Soman Challenge on GABAergic Transmission: Potential Mechanisms and Toxicological Relevance. In severely intoxicated guinea pigs, the IPSC frequency recorded from CA1 pyramidal neurons at 1 h after soman challenge was significantly lower than control. Because the IPSC amplitudes remained unchanged, the decreased IPSC frequency may have resulted from reduced firing of GABAergic interneurons synapsing onto the pyramidal neurons. The acute reduction of inhibitory inputs to CA1 pyramidal neurons can have a dual causal-consequential relationship with the convulsions the animals presented. Disinhibition of the pyramidal neurons can lead to development of seizures (Trevelyan, 2009), which can trigger loss of CA1 interneurons (Nadler, 1981; Ben-Ari, 1985) and further reduce inhibitory inputs to pyramidal cells. In fact, CA1 interneurons are highly vulnerable to seizure-induced death (Nadler, 1981; Ben-Ari, 1985), and neurodegeneration is always present in the hippocampus of animals severely intoxicated with soman (Filliat et al., 1999; Myhrer et al., 2005; Albuquerque et al., 2006).

The hippocampus receives cholinergic innervation from the basal forebrain nuclei (Woolf, 1991). Muscarinic receptors, particularly M1, are present in the somatodendritic region and/or along the axons of most CA1 interneurons (Behrends and ten Bruggencate, 1993; Cobb and Davies, 2005). Different types of CA1 interneurons also express somatodendritic $\alpha 7$ and $\alpha 4\beta 2$ nAChRs and receive excitatory innervation from glutamatergic neurons that express $\alpha 3\beta 4$ nAChRs (Albuquerque et al., 2009; Alkondon et al., 2009). Activation of these mAChRs and nAChRs, which are known to contribute to the acute toxicity of soman (Hassel, 2006), increases neuronal firing. Thus, the reduced frequency of IPSCs in pyramidal neurons of soman-exposed guinea pigs could be explained by reduced activity/expression of mAChRs and/or nAChRs on interneurons that synapse onto the pyramidal cells. A long-lasting reduction of mAChR expression has been observed in the hippocampus of rats exposed to a single convulsive dose of soman (Churchill et al., 1990). Likewise, a sustained reduction of $\alpha 3\beta 4$ nAChR-dependent excitation of CA1 stratum radiatum interneurons has been seen in the hippocampus of guinea pigs severely intoxicated with soman (Alkondon et al., 2009).

In mildly intoxicated guinea pigs, the IPSC amplitudes recorded at 1 h after soman challenge were significantly larger than control. Because the frequency of events was not altered, the increased IPSC amplitude could have resulted

from a presynaptic action that increases GABA release. Activation of M2 mAChRs present on presynaptic terminals of CA1 interneurons (Hajos et al., 1998) decreases quantal GABA release (Fukudome et al., 2004). Thus, desensitization of these receptors by excess ACh can facilitate synaptic release of GABA and increase the amplitude without affecting the frequency of IPSCs. Previous studies have proposed that animals will not present convulsions when exposed to soman if brain AChE inhibition does not reach at least 65% (Tonduli et al., 1999). Therefore, the degree of AChE inhibition in the hippocampus of mildly intoxicated guinea pigs may have been too low to cause sufficient build-up of ACh to overstimulate mAChRs and/or nAChRs. However, a small, yet persistent, rise in the ACh concentration can be sufficient to desensitize some of these receptor subtypes (Alkondon et al., 2000).

AChE-unrelated mechanisms can also contribute to the increased synaptic release of GABA onto CA1 pyramidal neurons. For example, soman inhibits adenosine A1 receptors with high affinity (Lau et al., 1991). These receptors are present in axon terminals of CA1 interneurons and their activation decreases GABA release (Jeong et al., 2003). Thus, soman-induced inhibition of A1 receptors would increase the amplitude without affecting the frequency of IPSCs. One cannot rule out the possibility that an increase in the number of postsynaptic GABA_A receptors contributes to the increased IPSC amplitudes.

Regardless of the severity of the signs of acute intoxication presented by the guinea pigs, the frequency of IPSCs recorded at 24 h after soman challenge was significantly lower than control. Because AChE activity remains significantly inhibited by 24 h after an exposure of guinea pigs to $1 \times LD_{50}$ soman (Lintern et al., 1998), the low frequency of IPSCs could be explained by ACh-induced desensitization of mAChRs and nAChRs on CA1 interneurons. Long-lasting loss of mAChR function caused by desensitization by excess ACh has been observed on the papillary sphincter muscle of rats exposed to low levels of soman (Dabisch et al., 2007). In addition, the decreased $\alpha 7$ nAChR activity recorded from CA1 stratum oriens interneurons at 24 h after an acute exposure of guinea pigs to $1 \times LD_{50}$ soman has been attributed to receptor desensitization by accumulated ACh (Alkondon et al., 2009).

Although the frequency of IPSCs returned to control levels by 6 to 9 days after soman challenge, their amplitudes became larger than control. Because a large portion of AChE activity is recovered by 7 days after soman exposure (Lintern et al., 1998), compensatory mechanisms may have overcome the decreased inhibitory input to the pyramidal neurons. Drugs that increase GABA_A receptor activity increase the expression of GABA_B receptors (Raol et al., 2005), which exert a presynaptic autoinhibitory effect on GABA release (Brown and Sihra, 2008). Soman-induced suppression of GABAergic transmission in the CA1 field of the guinea pig hippocampus can lead to a reduction of presynaptic GABA_B receptors and, consequently, disinhibit the synaptic release of GABA.

Soman-induced immediate and delayed changes in inhibitory inputs to CA1 pyramidal neurons can disrupt the hippocampal network oscillations that are essential for cognitive processing (Buzsáki, 2001), and, thereby, account for the

delayed cognitive dysfunctions triggered by this nerve agent (Wolthuis and Vanwersch, 1984; Myhrer et al., 2005).

Effectiveness of Galantamine as a Medical Countermeasure against Soman Intoxication. At 1.5 h after the treatment of guinea pigs with galantamine, the IPSC amplitudes were significantly larger than control. No concurrent change in the frequency of events was noted. At this time, brain concentrations of galantamine are within the range that favors its nicotinic allosteric potentiating action, in addition to a small degree of AChE inhibition (Albuquerque et al., 2006). Acting primarily as a nicotinic allosteric potentiating ligand, galantamine increases the frequency of ACh-induced IPSCs in rat hippocampal slices and human cerebral cortical slices in vitro (Santos et al., 2003). Such an effect could not have been detected in the slices taken from galantamine-treated guinea pigs, because the slices were maintained in galantamine-free ACSF and the nicotinic allosteric potentiating action of the drug is promptly reversible upon washout. The transient increase in IPSC amplitude in galantamine-treated guinea pigs, which was similar to that seen at 1 h in guinea pigs mildly intoxicated with soman, could be explained by the transient accumulation of ACh leading to M2 mAChR desensitization/internalization, which can last long after agonist removal (Krudewig et al., 2000).

Pretreatment of guinea pigs with galantamine prevented soman-induced immediate and delayed changes in inhibitory synaptic inputs to CA1 pyramidal neurons. A number of actions of galantamine could explain its effectiveness. First, reversible inhibition of brain AChE by galantamine may be sufficient to protect a significant pool of the enzyme from soman-induced irreversible inhibition, and, thereby, prevent excessive build-up of ACh concentrations. Second, the neuroprotective action of galantamine can prevent soman-induced neuronal loss, which does not seem to be exclusively caused by the seizures induced by the nerve agent (Filliat et al., 1999). Third, as a nicotinic allosteric potentiating ligand, galantamine can increase the activity of nAChR-associated signaling mechanisms (Albuquerque et al., 2009) to prevent changes in gene expression that may contribute to the delayed effects of soman on synaptic transmission.

In conclusion, pretreatment of guinea pigs with galantamine effectively counteracts the immediate and delayed effects of a lethal dose of soman on inhibitory synaptic transmission impinging onto CA1 pyramidal neurons. Considering that inhibitory inputs to CA1 pyramidal neurons control hippocampal rhythms that are essential for cognitive processing (Buzsáki, 2001), galantamine may be an effective medical countermeasure to prevent delayed cognitive dysfunctions triggered by an exposure to soman.

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References

- Albuquerque EX, Adler M, and Pereira EFR (2005) inventors; University of Maryland, Baltimore, assignee. Method of treating organophosphorous poisoning. International Patent Application PCT/US05/33789. 2005 Sept 23.
- Albuquerque EX, Deshpande SS, Kawabuchi M, Aracava Y, Idriess M, Rickett DL, and Boyne AF (1985) Multiple actions of anticholinesterase agents on chemosensitive synapses: molecular basis for prophylaxis and treatment of organophosphate poisoning. *Fundam Appl Toxicol* 5:S182–S203.
- Albuquerque EX, Pereira EFR, Alkondon M, and Rogers SW (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev* 89:73–120.
- Albuquerque EX, Pereira EFR, Aracava Y, Fawcett WP, Oliveira M, Randall WR, Hamilton TA, Kan RK, Romano JA Jr, and Adler M (2006) Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents. *Proc Natl Acad Sci USA* 103:13220–13225.
- Alkondon M, Aracava Y, Pereira EFR, and Albuquerque EX (2009) A single in vivo application of cholinesterase inhibitors has neuron type-specific effects on nicotinic receptor activity in guinea pig hippocampus. *J Pharmacol Exp Ther* 328:69–82.
- Alkondon M, Pereira EFR, Almeida LEF, Randall WR, and Albuquerque EX (2000) Nicotine at concentrations found in cigarette smokers activates and desensitizes nicotinic acetylcholine receptors in CA1 interneurons of rat hippocampus. *Neuropharmacology* 39:2726–2739.
- Aracava Y, Pereira EFR, Akkerman M, Adler M, and Albuquerque EX (2009) Effectiveness of donepezil, rivastigmine, and ($\pm$)huperzine A in counteracting the acute toxicity of organophosphorus nerve agents: comparison with galantamine. *J Pharmacol Exp Ther* 331:1014–1024.
- Behrends JC and ten Bruggencate G (1993) Cholinergic modulation of synaptic inhibition in the guinea pig hippocampus in vitro: excitation of GABAergic interneurons and inhibition of GABA release. *J Neurophysiol* 69:626–629.
- Ben-Ari Y (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience* 14:375–403.
- Brown DA and Sihra TS (2008) Presynaptic signaling by heterotrimeric G-proteins. *Handb Exp Pharmacol* 184:207–260.
- Buzsáki G (2001) Hippocampal GABAergic interneurons: a physiological perspective. *Neurochem Res* 26:899–905.
- Churchill L, Pazdernik TL, Cross RS, Nelson SR, and Samson FE (1990) Soman- or kainic acid-induced convulsions decrease muscarinic receptors but not benzodiazepine receptors. *Neurotoxicology* 11:57–71.
- Cobb SR and Davies CH (2005) Cholinergic modulation of hippocampal cells and circuits. *J Physiol* 562:81–88.
- Dabisch PA, Horsmon MS, Muse WT, Mioduszewski RJ, and Thomson S (2007) Muscarinic receptor dysfunction induced by exposure to low levels of soman vapor. *Toxicol Sci* 100:281–289.
- Duysen EG, Li B, Xie W, Schopfer LM, Anderson RS, Broomfield CA, and Lockridge O (2001) Evidence for nonacetylcholinesterase targets of organophosphorus nerve agent: supersensitivity of acetylcholinesterase knockout mouse to VX lethality. *J Pharmacol Exp Ther* 299:528–535.
- Filliat P, Baubichon D, Burckhardt MF, Pernot-Marino I, Foquin A, Masqueliez C, Perrichon C, Carpentier P, and Lallemand G (1999) Memory impairment after soman intoxication in rat: correlation with central neuropathology. Improvement with anticholinergic and antilutamatergic therapeutics. *Neurotoxicology* 20:535–549.
- Fosbraey P, Wetherell JR, and French MC (1990) Neurotransmitter changes in guinea-pig brain regions following soman intoxication. *J Neurochem* 54:72–79.
- Fukudome Y, Ohno-Shosaku T, Matsui M, Omori Y, Fukaya M, Tsubokawa H, Taketo MM, Watanabe M, Manabe T, and Kano M (2004) Two distinct classes of muscarinic action on hippocampal inhibitory synapses: M2-mediated direct suppression and M1/M3-mediated indirect suppression through endocannabinoid signaling. *Eur J Neurosci* 19:2682–2692.
- Gullapalli RP, Aracava Y, Zhuo J, Helal Neto E, Wang J, Makris G, Merchenthaler I, Pereira EFR, and Albuquerque EX (2010) Magnetic resonance imaging reveals that galantamine prevents structural brain damage induced by an acute exposure of guinea pigs to soman. *Neurotoxicology* 31:67–76.
- Hajós N, Papp EC, Acsády L, Levey AI, and Freund TF (1998) Distinct interneuron types express m2 muscarinic receptor immunoreactivity on their dendrites or axon terminals in the hippocampus. *Neuroscience* 82:355–376.
- Hassel B (2006) Nicotinic mechanisms contribute to soman-induced symptoms and lethality. *Neurotoxicology* 27:501–507.
- Huff RA, Corcoran JJ, Anderson JK, and Abou-Donia MB (1994) Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. *J Pharmacol Exp Ther* 269:329–335.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed, Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, Washington, DC.
- Jeong HJ, Jang IS, Nabekura J, and Akaike N (2003) Adenosine A1 receptor-mediated presynaptic inhibition of GABAergic transmission in immature rat hippocampal CA1 neurons. *J Neurophysiol* 89:1214–1222.
- Krudewig R, Langer B, Vögler O, Marksches N, Erl M, Jakobs KH, and van Koppen CJ (2000) Distinct internalization of M2 muscarinic acetylcholine receptors confers selective and long-lasting desensitization of signaling to phospholipase C. *J Neurochem* 74:1721–1730.
- Lau WM, Szilagyi M, and Freeman SE (1991) Effects of some organophosphorus compounds on the binding of a radioligand (8-cyclopentyl 1, 3-[3H]dipropylxanthine) to adenosine receptors in ovine cardiac membranes. *J Appl Toxicol* 11:411–414.
- Lintern MC, Wetherell JR, and Smith ME (1998) Differential recovery of acetylcholinesterase in guinea pig muscle and brain regions after soman treatment. *Hum Exp Toxicol* 17:157–162.
- Maelicke A and Albuquerque EX (2000) Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. *Eur J Pharmacol* 393:165–170.
- Myhrer T (2007) Neuronal structures involved in the induction and propagation of seizures caused by nerve agents: implications for medical treatment. *Toxicology* 239:1–14.
- Myhrer T, Andersen JM, Nguyen NH, and Aas P (2005) Soman-induced convulsions in rats terminated with pharmacological agents after 45 min: neuropathology and cognitive performance. *Neurotoxicology* 26:39–48.
- Nadler JV (1981) Minireview. Kainic acid as a tool for the study of temporal lobe epilepsy. *Life Sci* 29:2031–2042.
- Newmark J (2007) Nerve agents. *Neurologist* 13:20–32.
- Pereira EFR, Burt DR, Aracava Y, Kan RK, Hamilton TA, Romano JA Jr, Adler M,

- and Albuquerque EX (2008) Novel medical countermeasure for organophosphorus intoxication: connection to Alzheimer's disease and dementia, in *Chemical Warfare Agents* (Romano JA Jr, Lukey BJ, and Salem H eds) pp 219–232, CRC Press, Boca Raton, FL.
- Pereira EFR, Hilmas C, Santos MD, Alkondon M, Maelicke A, and Albuquerque EX (2002) Unconventional ligands and modulators of nicotinic receptors. *J Neurobiol* **53**:479–500.
- Raol YH, Zhang G, Budreck EC, and Brooks-Kayal AR (2005) Long-term effects of diazepam and phenobarbital treatment during development on GABA receptors, transporters and glutamic acid decarboxylase. *Neuroscience* **132**:399–407.
- Santos MD, Pereira EFR, Aracava Y, Castro NG, Fawcett WP, Randall WR, and Albuquerque EX (2003) Low concentrations of pyridostigmine prevents soman-induced inhibition of GABAergic transmission in the central nervous system: involvement of muscarinic receptors. *J Pharmacol Exp Ther* **304**:254–265.
- Shih TM and McDonough JH Jr (1997) Neurochemical mechanisms in soman-induced seizures. *J Appl Toxicol* **17**:255–264.
- Tonduli LS, Testylier G, Marino IP, and Lallement G (1999) Triggering of soman-induced seizures in rats: multiparametric analysis with special correlation between enzymatic, neurochemical and electrophysiological data. *J Neurosci Res* **58**:464–473.
- Trevelyan AJ (2009) The direct relationship between inhibitory currents and local field potentials. *J Neurosci* **29**:15299–15307.
- Wolthuis OL and Vanwersch RA (1984) Behavioral changes in the rat after low doses of cholinesterase inhibitors. *Fundam Appl Toxicol* **4**:S195–S208.
- Woolf NJ (1991) Cholinergic systems in the mammalian brain and spinal cord. *Prog Neurobiol* **37**:475–524.

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