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RESEARCH ARTICLE

Comparison of extracellular striatal acetylcholine and brain seizure activity following acute exposure to the nerve agents cyclosarin and tabun in freely moving guinea pigs

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Abstract

Organophosphorus nerve agents like cyclosarin and tabun are potent cholinesterase inhibitors. The inhibition of acetylcholinesterase, which is responsible for breaking down acetylcholine (ACh) at the synapse and neuromuscular junction, leads to a build-up of extracellular ACh and a series of toxic consequences including hypersecretion, tremor, convulsion/seizure, respiratory distress, coma, and death. This study employed simultaneous and continuous electroencephalographic recording and striatal microdialysis collection for quantification of ACh changes (via subsequent HPLC analysis) during acute exposure to a $1.0 \times LD_{50}$ subcutaneous dose of either cyclosarin or tabun to investigate differences in cholinergic and behavioral effects. Information about the unique mechanisms and consequences of different nerve agents is intended to aid in the development of broad-spectrum medical countermeasures for nerve agents. At the dose administered, non-seizure and sustained seizure responses were observed in both agent groups and in the tabun-exposed group some subjects experienced an unsustained seizure response. Significant extracellular ACh increases were only observed in seizure groups. Cyclosarin and tabun were found to exhibit some unique cholinergic and ictogenic characteristics. Lethality only occurred in subjects experiencing sustained seizure, and there was no difference in lethality between agent groups that progressed to sustained seizure.

Keywords: *Acetylcholine; acetylcholinesterase; cholinesterase inhibitors; cyclosarin; in vivo microdialysis; lethality; nerve agents; organophosphorus compounds; seizure activity; tabun.*

Introduction

Organophosphorus (OP) nerve agents, such as cyclosarin and tabun, inhibit cholinesterase (ChE) enzymes, a general category of enzymes including acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing acetylcholine (ACh) in the cholinergic synapses and neuromuscular junctions (Taylor 2001). Inhibition of AChE results in the accumulation of extracellular ACh within the synapses of both the central (CNS) and peripheral (PNS) nervous systems (Taylor 2001). The resulting hyperactivity of the cholinergic system can produce a sequence of toxic signs including hypersecretion, bronchoconstriction, miosis, muscular twitching, mental confusion, convulsive seizures, flaccid paralysis, respiratory distress, and death

(McDonough and Shih 1997; Ecobichon 2001; Taylor 2001; Eddleston et al. 2004b). The current US military treatment regimen for OP poisoning consists of administration of the muscarinic ACh receptor antagonist atropine sulfate, the oxime 2-PAM (pralidoxime; pyridine-2-aldoxime methylchloride), an AChE reactivator, and the benzodiazepine drug diazepam, a seizure and motor convulsant attenuator (Moore et al. 1995; Lallement et al. 1997; Taylor 2001; Eddleston et al. 2004a).

Through a series of pharmacological studies a triphasic model for nerve agent toxicity has been developed. In the event that the initial cholinergic phase leads to seizure, a second phase of cholinergic and non-cholinergic modulation follows and may eventually lead to a non-cholinergic

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phase in which anti-cholinergic drugs are no longer effective at terminating seizure (McDonough and Shih 1997; Shih and McDonough 1997). In several studies, homogenates of discrete brain regions taken from rats exposed to the nerve agent soman were analyzed for changes in enzymatic activity, and neurotransmitter and metabolite levels (Shih 1982; Fosbraey et al. 1990; Shih and McDonough 1997); one major limitation of these methods is the lack of distinction between intracellular and extracellular pools of analyte (i.e. ACh). The pharmacological consequences of soman have been investigated extensively, including several studies utilizing intracranial microdialysis in rats to measure changes in ACh during exposure (Lallement et al. 1992; Tonduli et al. 1999). In rats, soman intoxication caused a two-phase pattern of ACh release in the medial septum and hippocampus, while in the amygdala there was a steady rise to a maximum ACh release after 50 min (Lallement et al. 1992). Studies examining the neuropharmacological consequences of nerve agent exposure have focused primarily on soman, but given that the threat of exposure is just as great for other nerve agents like cyclosarin and tabun, or VX and sarin, reportedly deployed upon both soldiers and civilians (Malloy 2000; Szinicz 2005; Smart et al. 2008), additional investigation is warranted.

Differences in structure, metabolism, and physical properties of nerve agents can lead to unique toxic consequences and differing degrees of efficacy for a given therapeutic drug (for example oxime AChE reactivators) against a range of nerve agents (Shih et al. 2009a). As part of our continuing effort to develop improved broad-spectrum antidotes to treat nerve agent poisoning we have utilized simultaneous intracranial microdialysis and EEG recording to examine differences in cholinergic perturbations in the striatum among un-anesthetized, freely moving guinea pigs experiencing sustained, unsustained, or absent seizure response following exposure to different nerve agents. The striatum was chosen for its relatively large size (consistent probe placement) and high density of cholinergic interneurons (easily detectable ACh concentrations), and it serves as an excellent indicator of global cholinergic events while receiving afferent excitatory projections from brain regions directly involved in seizure initiation (Glenn et al. 1987; O'Donnell et al. 2010). We previously reported results from an earlier phase of this study in which the nerve agents sarin, soman, VX, and VR were investigated (O'Donnell et al. 2010). Here we present results obtained from the investigation of two additional OP nerve agents: cyclosarin and tabun (Figure 1).

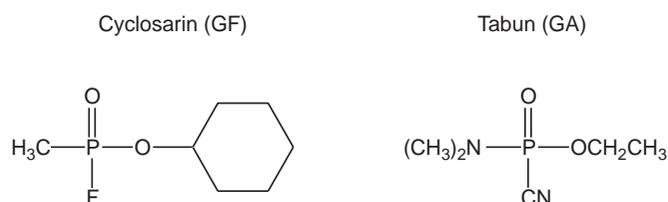


Figure 1. Chemical structures of the nerve agents cyclosarin and tabun.

Materials and methods

Subjects

Male Hartley guinea pigs (CrI:(HA) BR COBS) weighing 250–300 g were purchased from Charles River Labs (Kingston, NY). They were individually housed in polycarbonate cages in temperature ($21 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) controlled quarters that were maintained on a 12-h light–dark schedule (with lights on at 06:00 h). Guinea pig diet #7006 (Harlan Teklad, Madison, WI) and filtered tap water were freely available whenever the animals were in home cages. Animals were allowed to acclimate for 1 week prior to experimentation.

Research was conducted in compliance with the Animal Welfare Act of 1966 (P.L. 89-544), as amended and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996). The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC) of the US Army Medical Research Institute of Chemical Defense. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Materials

Chemicals/drugs

Atropine methyl nitrate (AMN), neostigmine bromide, ACh chloride, choline chloride, acetylthiocholine iodide, glucose, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl_2), calcium chloride (CaCl_2), 1-octanesulfonic acid sodium salt, Triton-X100, and monobasic sodium phosphate (NaH_2PO_4) were purchased from Sigma-Aldrich (St. Louis, MO). Reagent MB was obtained from ESA Biosciences, Inc. (Chelmsford, MA). Phosphoric acid (HPLC Grade), dibasic sodium phosphate (Na_2HPO_4), and Tris (hydroxymethyl) amino methane were purchased from Fischer Scientific (Fair Lawn, NJ). Saline (U.S.P.) was purchased from Braun Medical, Inc. (Irvine, CA). Heparin sodium was purchased from U.S.P., Inc. (Rockville, MD). Pentobarbital sodium and buprenorphine HCl were purchased from Ovation Pharmaceuticals (Deerfield, IL) and Reckitt Benckiser Pharmaceuticals, Inc. (Richmond, VA), respectively. Isoflurane liquid for inhalation was purchased from MINRAD, Inc. (Bethlehem, PA). Cyclosarin (GF; cyclohexyl methyl phosphonofluoridate) and tabun (GA; ethyl *N,N*-dimethyl phosphoramidocyanidate) were obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). DTNB (5, 5'-dithiobis(2-nitrobenzoic acid), bicinchoninic acid (BCA) Protein Assay Reagent A (sodium carbonate, sodium bicarbonate, BCA⁻ detection reagent, and sodium tartrate in 0.1 N sodium hydroxide), and BCA Protein Assay Reagent B (4% cupric sulfate) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). DTNB was prepared in Tris buffer (0.05 M, pH 8.2) to a concentration of 0.424 M.

Nerve agents and AMN were diluted in normal saline to a concentration to inject 0.5 ml/kg. For cyclosarin, the 2.0 ml aliquot stock solution was prepared in saline (vehicle), while for tabun it was prepared in multisol (40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, and 48% distilled deionized water). Both were stored at -80°C . Multisol maintains stability of the nerve agent tabun under storage conditions much better than saline as vehicle. On the day of experiment both cyclosarin and tabun were then diluted in saline and maintained on ice within 30 min prior to animal injections.

Equipment

Microdialysis guide cannulae (15 mm; part No. MD2250) and probes (BAS BR-2; 0.34 mm OD, part No. MD-2200) were purchased from Bioanalytical Systems (BAS) (W. Lafayette, IN). QND software and NE-4 amplifier were purchased from Neurodata, Inc. (Pasadena, CA). Kopf dual arm stereotaxic frame and Kopf electrode angle calibrator (model 935) were purchased from David Kopf Instruments (Tujunga, CA). Cortical electroencephalographic (EEG) screws were purchased from Plastics One, Inc. (Roanoke, VA), stainless steel wire from A-M Systems, Inc. (Sequim, WA), connectors from March Electronics, Inc. (Bohemia, NY), and dental acrylic from Lang Dental Mfg. Co., Inc. (Wheeling, IL). The high pressure liquid chromatography (HPLC) system consisted of a pump (Model 582), autosampler (Model 542), detector (Coulachem III), analytical column (ESA ACH-250 5- μm particle size, 250×3.2 mm), post-column reactor (ACH-SPR Part No. 70-0640), and analytical cell (Model 5040), all from ESA Biosciences, Inc. (Chelmsford, MA).

Surgery

Guinea pigs were anesthetized with isoflurane and surgically implanted with both cortical screw electrodes and a microdialysis guide cannula, using standard aseptic surgical techniques reported previously (Shih and McDonough 1999; O'Donnell et al. 2010). Briefly, guinea pigs were placed in the stereotaxic frame, and three cortical stainless-steel screw electrodes were implanted in the skull: two were placed bilaterally ~ 3.0 mm lateral from the midline and equidistant between bregma and lambda; the third was placed on the posterior calvaria as the reference electrode. Stainless-steel wires attached the screws to a miniature connector plug. The guide cannula was aimed at the striatum (+11.4 mm anterior, +3.6 mm lateral, -4.6 mm ventral to the skull surface) based on the atlas of Luparello (1967) using a zero co-ordinate. The screws, wires, connector, and guide cannula were then anchored to the skull with dental acrylic. The guinea pigs received 0.07 ml of 0.15 mg/ml buprenorphine HCl s.c. prior to and following surgery for pain control. The guinea pigs were allowed to recover for 6–10 days before the experiment began.

Experimental procedures

The techniques and timeline followed for microdialysis and EEG collection on the day of the experiment are the same as those employed for previous evaluation of sarin, soman,

VX, and VR (O'Donnell et al. 2010). On the day of the experiment, blood (0.25–0.5 ml) was drawn using the toe nail clip method (Vallejo-Freire 1951) for determination of baseline ChE activity. The animal was then placed in an individual sample collection chamber (23 cm deep \times 31 cm wide \times 45 cm high). The cortical screw electrodes were connected to an amplifier. EEG recordings were captured using QND software on a Macintosh personal computer (low frequency filter = 0.3 Hz; high frequency filter = 40 Hz; sampling rate = 128 Hz) and displayed on a monitor, as reported elsewhere (Shih and McDonough 1999; O'Donnell et al. 2010). The analog signal was amplified with an NE-4 amplifier before being processed on the Neurodata digital signal processor board. A brain microdialysis probe was inserted into the guide cannula, and perfused at a constant rate of 1.5 $\mu\text{l}/\text{min}$ with an artificial cerebrospinal fluid containing 140 mM NaCl, 3 mM KCl, 1 mM MgCl_2 , 1.2 mM CaCl_2 , 1.2 mM Na_2HPO_4 , 0.27 mM NaH_2PO_4 , 74 mM glucose, and 2 μM neostigmine bromide (pH 7.4). Animals were allowed to move freely during the course of the experiment, as the EEG/microdialysis tether allowed them to reach any area of the collection chamber. A 2-h normalization period was allowed to pass before commencing the continuous collection of dialysate samples at 15-min intervals (volume per sample = 22.5 μl) in 250- μl microcentrifuge tubes. Baseline dialysate samples were taken for 60 min (four 15-min fractions) prior to administering AMN, 1.0 mg/kg, intramuscularly (i.m.). AMN limits secretions of mucus and saliva to keep the airways clear to enhance survival during the collection period, but it does not cross the blood–brain barrier to interfere with CNS events. AMN was administered 30 min before a $1.0 \times \text{LD}_{50}$ subcutaneous (s.c.) dose of a nerve agent (cyclosarin, 57 $\mu\text{g}/\text{kg}$; or tabun, 120 $\mu\text{g}/\text{kg}$). Agent vehicle control animals (AMN/SAL) were administered AMN i.m. followed 30 min later by saline s.c. instead of nerve agent. A separate group of animals was treated with saline i.m. in place of AMN and 30 min later with another dose of saline s.c. to serve as the saline/saline (SAL/SAL) control group. In total, there were four separate treatment groups: a SAL/SAL control group, an AMN/SAL control group, and two AMN/nerve agent groups (cyclosarin or tabun). EEG recording and dialysate collection were continued for at least 210 min after nerve agent administration. The collected dialysate samples were stored at -20°C until analysis. At ~ 24 h post-exposure, a second blood sample was drawn for ChE analysis from the subjects that survived. Survivors were then deeply anesthetized with pentobarbital sodium solution (75–100 mg/kg, intraperitoneally [i.p.]) and perfused with saline followed by 10% neutral buffered formalin for verification of probe location.

ACh and choline (Ch) analysis

ACh and Ch levels within dialysate samples were quantified using isocratic HPLC with electrochemical (EC) detection. The autosampler injected 10 μl of each sample, and separation was achieved with the ESA analytical column at 27°C . The mobile phase (pH 8.0) was composed of anhydrous Na_2HPO_4 (100 mM), 1-octanesulfonic acid (2 mM), and an

anti-microbial agent Reagent MB (50 μ l per liter of deionized water) and was delivered at a constant rate of 0.350 ml/min. To facilitate EC detection, a post-column enzymatic reactor was utilized to convert ACh and Ch to detectable hydrogen peroxide (Lallement et al. 1992; Moor et al. 1998; Tonduli et al. 1999). The detector gain was set at 100 nA for Ch and 50 nA for ACh. The signal from the amperometric cell was delivered to a computer, and the digitized signal was then analyzed with EZchrom elite software (Scientific Software, Pleasanton, CA). The system was calibrated using a set of standards ranging from 0.63–10 pmoles of ACh and 1.07–25 pmoles of Ch. The typical retention time was 5.0 min for Ch and 7.6 min for ACh with excellent peak separation. These data were collected from all subjects (SAL/SAL $n=11$; AMN/SAL $n=15$; cyclosarin $n=16$; tabun $n=26$), with necessary instances of non-biased exclusion based on experimental error (insufficient sample volume, HPLC analysis error, etc.).

Blood collection and processing

Approximately 0.25–0.5 ml of blood was collected into a tube with 50 μ l of heparin sodium (15 units/ml) prior to nerve agent exposure, at the end of the microdialysis collection (270 min post-exposure), and then again from 24-h survivors. Blood was not collected from control animals at 270 min post-exposure, as the baseline and 24-h samples were considered sufficient to demonstrate steady ChE concentrations, and for the same reason blood was only collected from the first six AMN/SAL animals. For whole blood (WB) preparation, 20 μ l of collected blood was diluted (1:25) in 1% Triton-X100 (in saline) and mixed briefly. For red blood cell (RBC) samples, the remaining blood was centrifuged (5 min; 16,000 \times g), the plasma was removed, and 10 μ l of packed RBC were diluted (1:50) in 1% Triton-X100 (in saline) and mixed briefly. The diluted WB and RBC samples were frozen at -80°C until analyzed for ChE activity. Blood was not collected from deceased or extremely moribund animals.

ChE activity

ChE activity was measured in a spectrophotometer using the colorimetric method of Ellman et al. (1961) modified for use with a microplate reader (Shih et al. 2005; 2009a; b). The WB and RBC ChE activities were determined in triplicate using DTNB (0.424 M) as the chromophore and a super-saturated concentration of acetylthiocholine (17.1 mM) as the substrate. A sample volume of 10 μ l was added to each well to place results within the optimal detectable range of the assay. The plate was read at 410 nm at 20-s intervals for 3.5 min using a Spectramax Plus 384 microplate reader (Molecular Devices Corporation, Sunnyvale, CA), and the activity ($\mu\text{mol}/\text{min}/\text{ml}$) was determined using Softmax Plus 4.3 LS software (Molecular Devices Corporation). To supplement low sample size due to a lack of blood collected from the moribund and deceased, available blood from animals excluded from neurotransmitter analysis (due to microdialysis and HPLC specific experimental error) was included (SAL/SAL $n=11$; AMN/SAL $n=6$; cyclosarin $n=12$; tabun $n=33$).

Statistical analysis

All results are expressed as mean values \pm SEM. Blood ChE data were analyzed via a univariate general linear model with a type I sum of squares (fixed factor-group; covariant-baseline; dependent-exposure). Comparison of groups according to seizure occurrence and mortality within seizure groups was made using a cross-tabulated Chi-square test followed by pair-wise comparisons with Fisher's exact test. Seizure onset times were compared using a one-factor ANOVA. Raw baseline ACh and Ch concentrations (first four 15-min samples before AMN administration) for the control and experimental groups were subjected to ANOVA with repeated measures to test for similar cholinergic starting conditions. The post-exposure values of each subject were taken as a percentage of that subject's average baseline concentration, and these values were used for statistical analyses to reduce error and focus on time-course changes. It was necessary to perform a natural log transform of the data to meet the homogeneity of variance assumption. First, SAL/SAL and AMN/SAL groups were compared to examine the effect of AMN, followed by separate analyses in which sustained seizure, unsustained seizure, and non-seizure groups were compared to the AMN/SAL group for each agent. Analyses were performed with repeated measure ANOVA followed by one-way ANOVA to compare nerve agents at each observation time. Post-hoc analysis was done using Tukey's multiple comparison test. In all cases, significance was set at $p \leq 0.05$. All tests were run using SPSS (PASW) 17 for Windows.

Results

Seizure occurrence and lethality

EEG recordings were produced throughout the experiment and used to categorize animals into one of three groups: sustained seizures, which developed and persisted throughout the post-exposure collection period; unsustained seizures that developed to an EEG power similar to sustained seizure but terminated within 1 h of onset; and no seizures. Seizure onset was operationally defined as the appearance of ≥ 10 s of rhythmic high amplitude spikes or sharp wave activity in the EEG. Overall seizure occurrence rates (including sustained and unsustained) for cyclosarin and tabun were significantly different at 93.8% (15/16) and 53.8% (14/26), respectively, as were their sustained seizure rates at 93.8% (15/16) and 30.8% (8/26), respectively ($\chi^2=15.96$, $df=2$, $p<0.01$). The unsustained seizure occurrence rate, which was observed only in animals that received tabun, was 23.1% (6/26). The overall mortality rate was 43.8% (7/16) for cyclosarin and 15.4% (4/26) for tabun. Mortality was observed only in animals that experienced sustained seizures. Although seizure occurrence was more prevalent in the cyclosarin group than in the tabun group, both nerve agent groups showed similar lethality among sustained seizure animals. Twenty-four-hour mortality rates among animals in the sustained seizure group were 46.7% (7/15) for cyclosarin and 50% (4/8) for tabun, and were not significantly different.

Average sustained seizure onset times were 13 ± 1.2 min ($n=15$) for cyclosarin and 15 ± 1.8 min ($n=8$) for tabun. The unsustained seizure group exposed to tabun had a slightly longer average latency to seizure onset at 26 ± 7.7 min ($n=6$), but was significantly later compared to the sustained seizure group ($p < 0.05$). The average duration of an unsustained seizure was 15 ± 4.5 min ($n=6$).

Blood ChE activity

Average baseline ChE activities for blood were 1.62 and 2.58 $\mu\text{mol}/\text{min}/\text{ml}$ for RBC and WB, respectively. All nerve agent-exposed groups showed significant RBC AChE decreases (p 's < 0.001) to $\sim 8\%$ of baseline 270 min post-exposure and showed no significant change over the next 24 h (Table 1). Both SAL/SAL and AMN/SAL control groups showed no significant changes in ChE activity. Additionally, post-exposure WB total ChE was significantly inhibited in tabun and cyclosarin groups (p 's < 0.001) when compared to their baseline values and the control groups. The agent-exposed seizure and non-seizure groups showed no significant difference in ChE and AChE activity in WB and RBC.

Extracellular striatal ACh and Ch concentrations

Basal extracellular striatal ACh concentrations ranged between 0.28–7.10 $\text{pmol}/10 \mu\text{l}$. There were no significant differences between basal concentrations of individual treatment groups. Figure 2 shows the changes in ACh concentrations in seizure and non-seizure groups after exposure to $1.0 \times \text{LD}_{50}$ of cyclosarin (2A) or tabun (2B). Ch levels were highly variable, as previously reported for other nerve agents (O'Donnell et al. 2010). Additionally, nerve agent exposure did not significantly alter Ch levels in a consistent manner.

There were no significant changes in SAL/SAL ACh levels throughout the experimental period. The ACh levels of the AMN/SAL group were significantly elevated relative to the

SAL/SAL group from 45–90 min after the sham agent injection, as indicated in Figure 2a. All agent-exposed groups were compared to the AMN/SAL control group. The ACh levels for the cyclosarin sustained seizure group displayed a significant increase ($293.8 \pm 22.7\%$) 45 min after the nerve agent injection and reached its maximal increase ($363.5 \pm 31.3\%$) at 60 min after nerve-agent exposure. Significant elevation of the cyclosarin sustained seizure group relative to the control AMN/SAL group continued until 180 min after exposure. Because there was only one cyclosarin non-seizing animal, statistical analysis could not be performed on the non-seizure group.

The tabun sustained seizure group (Figure 2b) showed a significant increase in ACh in comparison to the AMN/SAL group. ACh levels started increasing at 60 min after agent injection ($322.1 \pm 53.5\%$) remained significantly elevated for 45 min, and were maximal ($344.7 \pm 79.4\%$) at 75 min after agent exposure. After a brief lull, sustained seizure group ACh levels were again significantly elevated at 135 min after tabun administration. Although the ACh levels for the unsustained seizure and non-seizure groups were not significantly different from those of the AMN/SAL group, it should be noted that at 60 min after agent exposure the difference between the AMN/SAL and unsustained seizure groups was very close to the arbitrary level of significance ($p = 0.070$).

Discussion

Utilizing intracranial microdialysis and simultaneous EEG recording, we have continued our investigation of the cholinergic events taking place in the CNS after exposure to various OP nerve agents in unanesthetized, freely moving guinea pigs. Within each nerve agent group, subjects were separated according to seizure occurrence to examine the consequences of the different responses. Both sustained

Table 1. ChE activity in RBC and WB.

Treatment group	n	Average RBC AChE $\mu\text{mol}/\text{min}/\text{ml}$ (% baseline)			WB total ChE $\mu\text{mol}/\text{min}/\text{ml}$ (% baseline)		
		Baseline	270 min	24-h	Baseline	270 min	24-h
			post-exposure	post-exposure		post-exposure	post-exposure
Saline/saline	11	2.41 ± 0.07 (100%)	n/a	2.33 ± 0.10 (96.8% \pm 4.2)	2.42 ± 0.12 (100%)	n/a	2.33 ± 0.11 (97.2% \pm 3.6)
AMN/saline	6	2.22 ± 0.21 (100%)	n/a	2.38 ± 0.21 (107.7% \pm 4.9)	2.25 ± 0.14 (100%)	n/a	2.27 ± 0.16 (101.5% \pm 3.7)
Cyclosarin sustained seizure	11	2.28 ± 0.08 (100%)	0.14 ± 0.01 (6.1% \pm 0.6)	0.32 ± 0.11 (13.3% \pm 4.3)	2.17 ± 0.06 (100%)	0.36 ± 0.02 (16.6% \pm 0.8)	0.52 ± 0.04 (24.3% \pm 2.3)
Cyclosarin non-seizure	1	$2.58 \pm \text{n/a}$ (100%)	$0.21 \pm \text{n/a}$ (8.0% \pm n/a)	$0.25 \pm \text{n/a}$ (9.8% \pm n/a)	$2.27 \pm \text{n/a}$ (100%)	$0.42 \pm \text{n/a}$ (18.3% \pm n/a)	$0.5 \pm \text{n/a}$ (22.1% \pm n/a)
Tabun sustained seizure	7	2.01 ± 0.33 (100%)	0.16 ± 0.06 (7.6% \pm 2.0)	0.09 ± 0.020 (6.8% \pm 3.4)	2.08 ± 0.16 (100%)	0.31 ± 0.05 (14.8% \pm 2.3)	0.34 ± 0.06 (16.1% \pm 2.7)
Tabun unsustained seizure	7	1.62 ± 0.14 (100%)	0.05 ± 0.02 (3.2% \pm 1.3)	0.06 ± 0.02 (3.4% \pm 1.0)	1.68 ± 0.14 (100%)	0.26 ± 0.04 (15.1% \pm 1.7)	0.40 ± 0.04 (23.1% \pm 1.5)
Tabun non-seizure	17	2.12 ± 0.12 (100%)	0.12 ± 0.20 (5.7% \pm 1.0)	0.11 ± 0.02 (5.2% \pm 0.7)	2.10 ± 0.11 (100%)	0.39 ± 0.04 (18.3% \pm 1.3)	0.54 ± 0.05 (24.9% \pm 1.3)

Blood from each guinea pig was collected prior to experiment and separated into RBC and WB for baseline ChE activity analysis. At 270 min and 24-h after cyclosarin or tabun exposure, blood was taken again for determination of RBC and WB ChE activity. ChE activity was expressed as mean $\mu\text{mol}/\text{min}/\text{ml} \pm \text{SEM}$ and as the mean of the % of each animal's baseline activity $\pm \text{SEM}$.

n/a = not applicable; Blood was not collected at 270 min for the control groups. SEM could not be calculated for the cyclosarin non-seizure group because $n=1$.

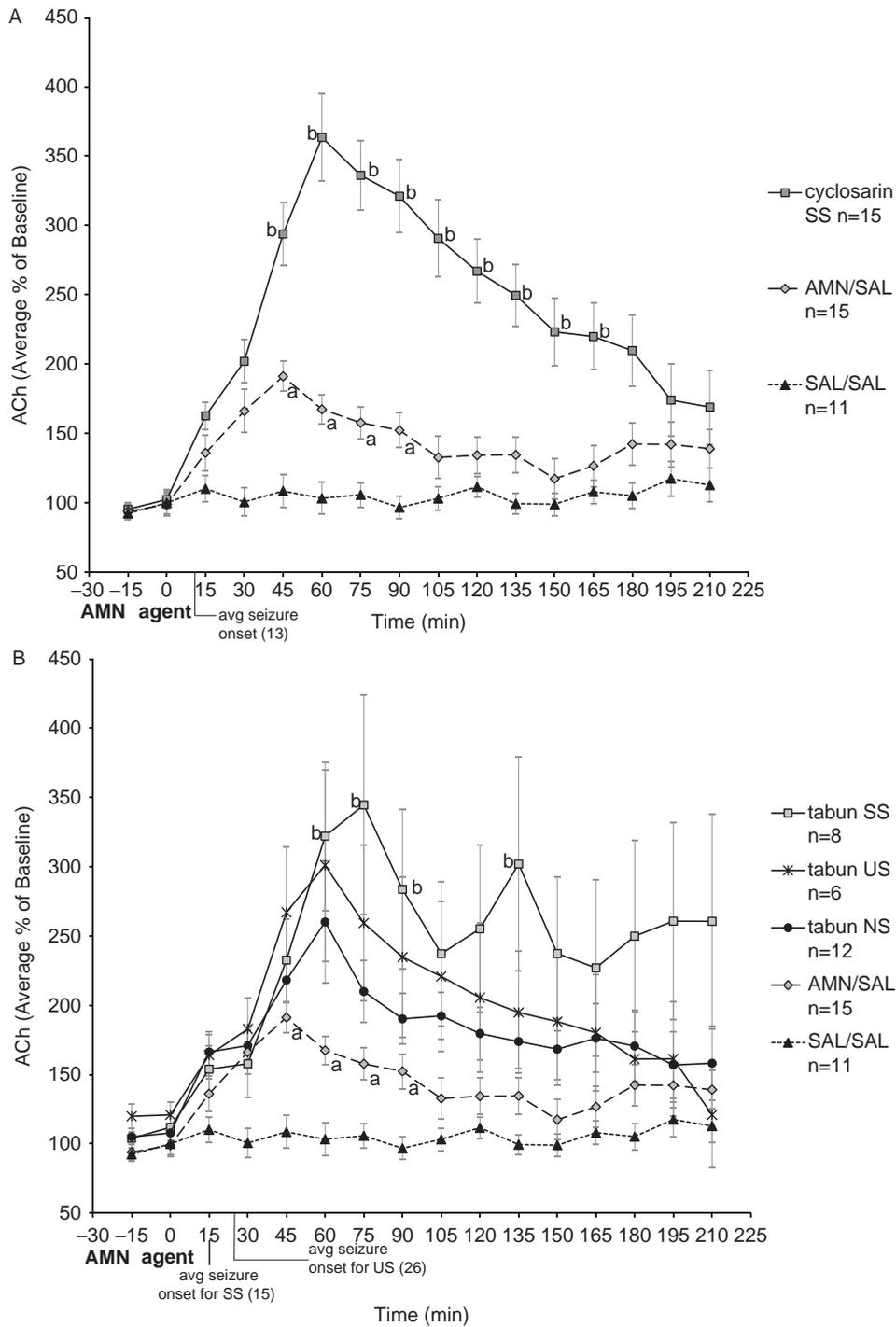


Figure 2. Changes in extracellular striatal ACh concentrations after exposure to $1 \times LD_{50}$ of cyclosarin (a) or tabun (b). ACh concentrations were normalized as a percentage of each subject's average baseline, and the points in the figure represent the average % baseline within each response group at each time point (error bars: \pm SEM). Times of AMN and nerve agent injections are indicated on the x-axis, as well as average seizure onset times. SS=sustained seizure; US=unsustained seizure; and NS=non-seizure. ^a $p < 0.05$ vs SAL/SAL. ^b $p < 0.05$ vs AMN/SAL. (See colour version of this figure online at www.informahealthcare.com/txm).

seizure and non-seizure responses resulted from exposure to a $1.0 \times LD_{50}$ dose of nerve agent, as previously observed in groups of guinea pigs exposed to sarin, soman, VX, or VR (O'Donnell et al. 2010), in which a few anomalous incidences of unsustained seizure were also observed and grouped along with sustained seizure results. However, the response to tabun was unique in that a higher occurrence of

unsustained, self-terminating seizure was observed (23.1%), which allowed for comparison of the ACh profiles of three EEG response groups for this nerve agent. Neither RBC AChE nor total ChE in whole blood was dependent on seizure occurrence. This is in agreement with previous results (O'Donnell et al. 2010), and is not surprising given that blood ChE is of little physiological consequence and immediately

available to the nerve agent after exposure. It should be noted that 50% lethality was sometimes not observed in this model because the $1.0 \times LD_{50}$ doses of nerve agent used were not determined in conjunction with administration of the peripherally selective drug AMN. Therefore, this model may highlight differences between the peripheral-to-central toxicity ratios of each nerve agent.

Among subjects experiencing sustained seizure, significant elevations of ACh above control were observed. Increases in extracellular striatal ACh after nerve agent exposure coincide with the timing of previously reported striatal AChE inhibition after exposure to $1.0 \times LD_{50}$ cyclosarin and tabun (Shih et al. 2005). However, seizure initiation preceded significant elevations of ACh by an average of 32 and 45 min for the cyclosarin and tabun groups, respectively. Prior to and immediately following seizure initiation, ACh concentrations among seizure animals are essentially indistinguishable from those of the non-seizure animals. These observations are consistent with sarin, soman, VX, and VR data (O'Donnell et al. 2010) and indicate that although elevated ACh creates seizurogenic conditions, additional variables that were not measured in this study are likely necessary for triggering seizure. By examining the differential response to a given dose of nerve agent in this and similar models, other researchers have identified non-cholinergic pathways of interest in ictogenesis.

Stress response and adaptation, involving a myriad of signaling pathways, have been implicated as key factors in determining the likelihood of ictogenesis after exposure to a seizurogenic ED_{50} (median effective dose) of nerve agent. Tonduli et al. (1999) exposed rats to an ED_{50} of soman and performed power spectrum analysis of the EEG recordings to look for a significant correlation between waveforms and seizure response. They found that only rats lacking an increase in the gamma band developed seizures. They concluded that, in addition to elevated ACh, stress response contributed to seizure initiation, as the gamma band is associated with the noradrenergic pathways of the sympathetic stress response, and an increase in the gamma band could represent an adaptation response to alleviate the negative effects of a stressor. Additional indices of stress response can be found in behavioral observations and efficacy of anti-convulsant drugs after nerve agent exposure. Reductions in GABA-stimulated chloride uptake due to reduction in GABA_A receptor activity are associated with response to an uncontrollable stressor, and excessive chewing, which we commonly observe in animals recently exposed to nerve agent, is a coping behavior associated with alleviating the GABAergic perturbations of the stress response (Martijena et al. 2002). Benzodiazepines, in particular midazolam, have demonstrated rapid termination of nerve agent-induced seizures (Shih et al. 2003), and their primary mechanism of action is allosteric modulation of the GABA_A receptor, which increases the receptor's affinity for binding the inhibitory neurotransmitter GABA. These observations highlight the utility of non-cholinergic drugs like benzodiazepines in the early stages of nerve agent toxicity as a supplement to the various avenues of cholinergic intervention (i.e. carbamate pre-treatments, anti-cholinergics, oxime

reactivators). Although benzodiazepines are effective anti-convulsants, the possibility of adverse side-effects warrants further investigation into the relationship between stress and seizure susceptibility to identify new therapeutic targets to elicit more specific anti-convulsant effects that would benefit chemical defense efforts as well as the broader field of epilepsy research.

The large increases in extracellular ACh observed after seizure initiation that were absent in non-seizure animals indicate that the seizure activity itself contributed to additional ACh release. This is further supported by ACh elevation observed in the tabun unsustained seizure group that was similar to that of the sustained seizure group until shortly after seizure self-termination, at which point a decline in ACh to non-seizure levels can be observed. The mechanism of seizure self-termination is unclear. Considering possible agent-specific effects, the high rate of unsustained seizure in the tabun-exposed group may be due to a lower probability of transitioning beyond the initial cholinergic phase into the later excitatory amino acid (EAA) phase of toxicity for lack of sufficient ACh driven potentiation of glutamatergic NMDA (*N*-methyl *D*-aspartate) receptors (Markram and Segal 1990) as compared to the cyclosarin-exposed group. As evidence, the sustained seizure groups of both agents exhibited 30 min of elevated ACh significantly greater than control before ACh began to decline towards baseline concentrations (during which significant ACh elevations were still observed), while the unsustained seizure group only approached significantly elevated levels during a single 15-min period before declining towards baseline concentrations. Considering non-agent-specific effects, lower excitability resulting from more efficient stress response attenuation relative to the tabun-exposed sustained seizure group may reduce the additional ACh released due to seizure in the unsustained seizure group. Therefore, the severity of cholinergic toxicity determines the probability of ictogenesis and later the probability of transition to the EAA phase, but both events are influenced by non-cholinergic modulation likely related to stress response and adaptation. Furthermore, these data indicate that the transition from cholinergic to EAA seizure maintenance occurs after 30–75 min of seizure activity, consistent with anti-convulsant drug studies indicating a transition to predominantly non-cholinergic seizure maintenance at that time (McDonough and Shih 1997).

Trends in the tabun non-seizure group may be due at least in part to an AMN effect, since the AMN/saline group did show significant ACh increases compared to the saline/saline group. The effects of AMN are predominantly peripheral, as it does not readily cross the blood–brain barrier (Chambers and Chambers 1989; Shih 1991; Maalouf et al. 1998), allowing for focused evaluation of CNS-induced lethality. The observed effect may be due to disruption of the blood–brain barrier by the microdialysis probe (Allen et al. 1992; Westergren et al. 1995; Groothuis et al. 1998), allowing AMN localized passage into the sampling area, although the mechanisms resulting in increased extracellular ACh concentrations are unknown. The possibility of such a disruption necessitates a sufficient

recovery period between probing and sample collection (O'Donnell et al. 2010).

Lethality was dependent on sustained seizure activity, since it was only observed in subjects that experienced sustained seizure. This agrees with sarin, soman, VX, and VR data (O'Donnell et al. 2010) and highlights the importance of controlling seizure in reducing lethality (Shih et al. 2003). Cyclosarin had a significantly higher propensity for causing sustained seizures, but the lethality rates in sustained seizure groups for cyclosarin and tabun exposure were nearly identical. We previously observed higher seizure likelihood in animals exposed to VX and VR compared to those exposed to sarin, but in agreement with the present study, lethality among seizure animals was not significantly different (O'Donnell et al. 2010). No lethality was observed in the tabun-exposed unsustained seizure group that likely failed to transition into the non-cholinergic phase of seizure. This is in agreement with the existing model of nerve agent toxicity given that the final non-cholinergic phase in which excitatory amino acids take over seizure maintenance largely contributes to lethality by way of excitotoxic damage (McDonough and Shih 1997; Shih and McDonough 1997). Thus, cyclosarin and tabun display unique propensities for seizure initiation and for transition into sustained non-cholinergic seizure, but lethality of sustained seizures appears to be independent of the agent-specific events leading up to the excitatory amino acid transition. However, this is not to say that unique nerve agent characteristics can be ignored when considering therapeutic intervention after seizure initiation, as some drugs target the agent-enzyme complex itself. Centrally acting tertiary oximes terminate seizure with varying degrees of efficacy according to the nerve agent involved (Shih et al. 2009b; Skovira et al. 2010), and differences in agent distribution as well as unique characteristics of agent-enzyme complexes influence that efficacy. The experimental model employed in this study could offer insightful endpoints in future studies investigating the therapeutic window of tertiary oximes as it relates to the cholinergic phase of toxicity, and ACh levels after seizure termination would serve as a useful metric for quantifying the rate of hydrolysis of excess ACh after enzyme reactivation in different brain regions.

Our evaluation of cyclosarin and tabun, taken as a whole with our previous evaluation of sarin, soman, VX, and VR (O'Donnell et al. 2010), provides insight into the similarities and differences of these OP nerve agents. There was no difference in blood ChE activity among the response groups of any of the nerve agents. At an LD₅₀ dose, the G-type agents (sarin, soman, cyclosarin, and tabun) exhibited similar seizure latency times, while the V-type agents (VX and VR) exhibited later seizure onset, most likely due to impeded passage through the blood-brain barrier. Seizure initiation preceded ACh differences among response groups of each agent, suggesting that additional variables influenced the likelihood of ictogenesis. Large ACh increases in seizure groups after initiation and ACh decline after unsustained seizure termination indicate that seizure activity contributed to additional ACh release, but the pharmacological mechanism of this

phenomenon is currently unclear. In the sustained seizure groups, the eventual ACh trend back towards control levels (possibly due to release outpacing synthesis) while seizure persisted illustrated the transition to predominantly non-cholinergic, excitatory amino acid seizure maintenance, in agreement with the triphasic model of nerve agent toxicity (McDonough and Shih 1997; Shih and McDonough 1997). Cyclosarin was more likely than tabun to cause seizure, and those seizures were more likely to transition to the non-cholinergic phase, as opposed to self-terminating. Lethality was dependent on sustained seizure and, thus, progression beyond the early cholinergic phase. Once seizure had transitioned to the excitatory amino acid phase, unique characteristics of the nerve agents were no longer found to influence the prognosis, as no significant differences were observed among the mortality rates in the sustained seizure groups of each agent. The results obtained from investigating cyclosarin and tabun, taken along with previously reported sarin, soman, VX, and VR data (O'Donnell et al. 2010), detail the unique physiological responses resulting from exposure to different OP nerve agents. Differences in ACh profiles, seizure latency, and propensity to cause seizure among the nerve agents tested reveal the previously available, predominantly soman-influenced paradigm as insufficient, and provide an improved guide in our mission to develop optimally effective broad-spectrum medical countermeasures to mitigate the threat of chemical warfare nerve agents.

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