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Effects of physostigmine and human butyrylcholinesterase on acoustic startle reflex and prepulse inhibition in C57BL/6J mice☆

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Abstract

The use of exogenously administered cholinesterases as bioscavengers of highly toxic organophosphorus nerve agents is a viable prophylactic against this threat. To use this strategy, cholinesterases must provide protection without disrupting behavior when administered alone. To assess behavioral safety, the acoustic startle reflex and prepulse inhibition (PPI) of C57BL/6J mice were investigated following administration of human plasma-derived butyrylcholinesterase (HuBChE). Two hours before testing, four groups of mice ($n = 10$ per group) were pretreated with saline or HuBChE (2000 U, ip). Fifteen minutes before testing, subjects received either saline or the carbamate physostigmine (0.4 mg/kg, sc). Mice exposed to physostigmine exhibited a significant attenuation of the startle reflex, an increased time to peak startle amplitude, and significantly increased PPI. This effect was partially mitigated in mice pretreated with HuBChE. HuBChE alone did not change startle behavior or PPI significantly compared to saline controls. The circulatory time-course of butyrylcholinesterase was assessed in a separate group of mice and revealed levels approximately 600 times the physiological norm 2–4 h post administration. Thus, HuBChE does not appear to significantly alter startle or PPI behavior at a dose 30-fold higher than that estimated to be necessary for protection against $2LD_{50}$ of soman in humans.
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1. Introduction

Classic nerve agents, such as tabun (GA), sarin (GB), soman (GD) and VX, are extremely toxic organophosphorus (OP) compounds that pose a serious threat to both civilians and the military. These agents are believed to produce their lethal effects by irreversibly binding to the enzyme acetylcholinesterase (AChE), leading to acetylcholine (ACh) accumulation at synaptic sites and hyperactivity of the cholinergic system (Fonnum and Guttormsen, 1969; Shih, 1982). Left untreated, nerve agent poisoning may result in a rapid progression of symptoms, including convulsions, hypersecretion, and possibly death due to cardiovascular and respiratory failure (Dunn and Sidell, 1989; Shih, 1982). The current treatment for OP intoxication consists of a combination of compounds including a prophylactic carbamate, an antimuscarinic, an oxime nucleophile (reactivator), and an anticonvulsant under pre- and post-exposure conditions (Doctor et al., 1991; Lenz et al., 2001; Wilson and Ginsburg, 1955). Unfortunately, this therapy for nerve agent exposure fails to provide complete protection concomitant with side effects from some of the drugs employed (Castro et al., 1992; Leadbeater et al., 1985; McDonough, 2002; McDonough et al., 1989; McDonough and Shih, 1997; Wolthuis et al., 1989).

☆ The views expressed are those of the authors and should not be construed to represent the positions of the Department of the Army or Department of Defense (AR 360-5).

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While AChE inhibition is believed to be the primary mechanism of OP intoxication, OP chemical warfare agents and pesticides also bind other esterases in blood and tissue, including butyrylcholinesterase (BChE), carboxylesterase, and paraoxonase (for review see Lenz et al., 2001). Thus, an alternate approach to prevent OP lethality and minimize side effects involves exogenous administration of enzymes, such as cholinesterases (ChEs), as pretreatment drugs (Allon et al., 1998; Ashani et al., 1991; Broomfield et al., 1991; Doctor et al., 2001; Doctor et al., 1991; Maxwell et al., 1992; Raveh et al., 1989; Raveh et al., 1997; Wolfe et al., 1992; Wolfe et al., 1987). ChEs act to neutralize ChE-inhibiting compounds (e.g., OP nerve agents) in a stoichiometric manner before they reach physiological targets such as the brain (Ashani, 2000; Doctor et al., 2001; Doctor et al., 1991; Raveh et al., 1989; Raveh et al., 1997). A clear advantage of this approach over the current multiple-drug regimen is that a single agent may provide more than adequate protection against OP intoxication and death. Moreover, early evidence suggests that protection may be afforded in the absence of symptoms or behavioral side effects (Doctor et al., 2001; Lenz et al., 2001). Numerous studies have demonstrated that fetal bovine AChE provided significant protection against irreversible ChE-inhibiting OP compounds (e.g., MEPQ), and chemical warfare nerve agents (e.g., VX and GD) in nonhuman primates and mice (Maxwell, 1992; Raveh et al., 1989; Wolfe et al., 1992; Wolfe et al., 1987). Similarly, equine (Eq) and human (Hu) BChE were effective against various OP agents in mice, rats, guinea pigs and nonhuman primates (Allon et al., 1998; Ashani et al., 1991; Brandeis et al., 1993; Genovese and Doctor, 1995; Raveh et al., 1997; Raveh et al., 1993).

Recently, research has focused on the development of plasma-derived HuBChE as a bioscavenger for the protection of humans against nerve agent toxicity. HuBChE is a broadly applicable prophylactic antidote against OP-induced toxicity that should significantly improve an organism's ability to detoxify other naturally occurring and synthetic ChE inhibitors, including toxic doses of the carbamate physostigmine and OP pesticides (Ashani, 2000). While no study has examined the detoxification of short-acting cholinesterase inhibitors (e.g., toxic doses of carbamates including physostigmine), HuBChE was shown to provide significant protection against otherwise fatal exposure to tabun, sarin, soman, and VX in mice, rats, guinea pigs and nonhuman primates (Allon et al., 1998; Ashani et al., 1991; Brandeis et al., 1993; Raveh et al., 1997; Raveh et al., 1993). Also, a single dose of HuBChE in humans exhibited extended circulatory stability and reasonable bioavailability over a period of approximately 2–12 days (Garry et al., 1974; Jenkins et al., 1967; Ostergaard et al., 1988; Schuh, 1977; Stovner and Stadskleiv, 1976), suggesting a long window of protection in vivo.

HuBChE was chosen over Eq or other animal BChEs because the enzyme is intended for use in humans. Research indicates that there is increased antibody-mediated clearance following *heterologous* enzyme administration (e.g., using EqBChE in nonhuman primates or humans), especially following repeated enzyme administrations (Matzke et al., 1999; Maxwell et al., 1992). This is potentially problematic because antibody-mediated clearance will reduce the level of circulating enzyme, possibly below the therapeutic level. The level of circulating antibodies produced in response to heterologous and homologous ChEs, and the impact on enzyme clearance, was studied in macaques (Matzke et al., 1999; Maxwell et al., 1992; Rosenberg et al., 2002). These studies suggest that homologous ChE administration would produce high enzyme stability over a period of days with low immunogenicity. Thus, human enzymes like HuBChE have the best potential for use in humans because they could be employed repeatedly and more effectively.

Safety data regarding the effect of HuBChE on behavior is relatively limited. Brandeis et al. (1993) demonstrated that HuBChE is protective against exposure to soman, and that spatial memory, as assessed by a Morris water maze 1 week post-treatment, is unaffected by exogenous presentation of this enzyme. Similarly, Raveh et al. (1997) examined spatial discrimination in a limited sample of rhesus macaques exposed to both HuBChE and VX or soman. For subjects where the ratio of enzyme to OP was near or over 1 : 1, no or mild signs of toxicity were observed, largely with recovery by the next day. Regarding HuBChE safety specifically, four rhesus macaques were exposed to either 13 (10,400 units) or 34 mg (27,200 units) of HuBChE. No observable deficits were reported resulting from HuBChE administration alone. Studies of EqBChE in nonhuman primates (Matzke et al., 1999) also support the observations made by Raveh et al. Matzke et al. (1999) exposed four rhesus macaques to 27,000 and then to 54,000 units EqBChE, separated by 21 weeks. One hour following each enzyme administration, the macaques demonstrated no impairment on a six-item serial probe recognition task. Given that this enzyme is intended to be given as a prophylactic treatment (i.e., prior to or in the absence of nerve agent exposure), further research is necessary to fully characterize the behavioral toxicity, or lack thereof, of HuBChE. To eventually employ this strategy it is critical to confirm that HuBChE provides protection without disrupting behavior when administered in the absence of chemical warfare nerve agents.

A potentially valuable tool for evaluating the behavioral toxicity of HuBChE is the acoustic startle reflex. The acoustic startle reflex and prepulse inhibition (PPI) of that reflex are sensitive tools for investigating neurochemical modulation of sensorimotor processing and reflex excitability in rodents (Davis, 1980; Geyer et al., 2001). Specifically, the role of the cholinergic system in the acoustic startle response and PPI is not fully understood (Davis, 1980; Geyer et al., 2001). However, the acoustic startle reflex is sensitive to disruption by numerous ChE inhibitors, including heptylphysostigmine, physostigmine, pyridostigmine, tacrine and soman in a variety of species including rats, mice and guinea pigs (Haggerty et al., 1986;

Jones and Shannon, 2000b; Philippens et al., 1997; Philippens et al., 1996; Scremin et al., 2003; Servatius et al., 1998; Servatius et al., 2000; Waite and Thal, 1995). For example, Haggerty et al. (1986) observed systematic and dose-dependent decreases in acoustic startle reactivity in rats exposed to sublethal doses of soman. Similar results were observed by Jones and Shannon (2000b) with rats exposed to the ChE inhibitors physostigmine and tacrine. Because dose-dependent ChE inhibition decreases startle reactivity, it is possible that an overabundance of ChE might enhance startle reactivity.

Similarly, a strong role for the cholinergic system has been implicated in PPI behavior. Wu et al. (1993) demonstrated that choline-deficient rats exhibited decreased PPI that was partially reversed by the cholinergic agonist arecholine. Sipos et al. (2001) demonstrated that benactyzine, an anticholinergic agent that also competitively binds BChE (Bodur et al., 2001), dose-dependently decreased PPI in rats. Jones and Shannon (Jones and Shannon, 2000a; Jones and Shannon, 2000b) demonstrated that the cholinergic antagonists scopolamine, trihexyphenidyl, and benzotropine decreased PPI. Notably, they also exposed rats to the cholinesterase inhibitors tacrine (0.3–10 mg/kg) and physostigmine (0.01–0.1 mg/kg), but observed no alteration in PPI at the doses specified. Although PPI is sensitive to cholinergic modulation, the limited research with cholinesterase and cholinesterase inhibitors provides no clear prediction regarding the impact of exogenously administered physostigmine and butyrylcholinesterase on PPI.

The primary purpose of this study was to investigate the behavioral toxicity of a large dose of purified HuBChE on the acoustic startle reflex and prepulse inhibition of adult male C57BL/6J mice. It was expected that the subjects exposed to only HuBChE would not differ from saline controls, indicating a lack of a behavioral side effect, based on the previous research of Raveh et al. (1997). Previous research using ChE inhibitors indicated subjects exposed to physostigmine in the absence of HuBChE pretreatment should exhibit significantly depressed startle behavior relative to saline controls. If HuBChE does improve an organism's ability to detoxify ChE inhibitors like physostigmine as suggested by Ashani (2000), mice exposed to HuBChE and physostigmine should not exhibit altered startle behavior relative to HuBChE only controls, but their behavior should differ from those exposed to physostigmine alone. Data from this study will further clarify the impact of HuBChE and physostigmine on reflexive and PPI behavior.

2. Methods

This research was conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, 1996, and the Animal Welfare Act of 1966, as amended. The experimental protocol was reviewed and approved by the Walter Reed Army Institute of Research Institute Animal Care and Use Committee.

2.1. Subjects

The subjects were 61 male C57BL/6J mice and testing began when the mice were 12 weeks old. Four groups of ten mice ($N=40$) were used for the acoustic startle reflex and PPI assessment and 21 mice (saline–saline, $n=5$; saline–physostigmine, $n=5$; HBChE–physostigmine, $n=8$; HBChE–saline, $n=3$) were used to assess the time-course of butyrylcholinesterase. Mice were individually housed in a temperature- (21±1 °C) and humidity-controlled environment and maintained on a 12-h light/dark cycle with the light portion beginning at 0600. Food and water were available ad lib throughout the duration of the experiment, except in the experimental chamber.

2.2. Materials

HuBChE was purified from 120 kg of Cohn Fraction IV-4 paste after resuspension in 50 mM sodium phosphate, pH 8.0 buffer containing 1 mM EDTA. The enzyme was purified by affinity chromatography on a procainamide–Sepharose gel followed by ion-exchange chromatography on a DEAE Sepharose (fast-flow) column.

One milligram of pure enzyme contains 11 nmol active sites and has a specific activity of 700 units. Enzyme activity was assayed by the procedure of Ellman et al. (1961) with butyrylthiocholine as the substrate. Purified HuBChE and physostigmine hemisulfate (Sigma, St. Louis, MO, USA) were dissolved in sterile physiological saline. Physostigmine was prepared so that the final dose would be 0.4 mg/10 ml/kg (equal to 617 nmol/kg). The HuBChE was prepared so that the final dose was 2000 U/200 μl physiological saline per mouse.

2.3. Characterization of enzyme activity of exogenously administered HuBChE

To assess BChE activity in vivo, 21 total mice that were not behaviorally tested were injected with 2000 U of HuBChE or saline ip in a volume of 200 μl per mouse. Blood (10 μl) was sampled immediately before HuBChE injection and seven times post injection (1, 2, 4, 19, 27, 50, 72 and 168 h) from the tail vein. Mice from each group received either an injection of saline or physostigmine (sc) 15 min prior to the 2-h blood collection. The collected blood was diluted with deionized water at either 10 or 100× and was assayed using the procedure of Ellman et al. (1961) using butyrylthiocholine as the substrate.

2.4. Behavioral apparatus

The amplitude and time point of the acoustic startle reflex were recorded using a Startle Monitor System

(Hamilton Kinder, Poway, CA, USA) composed of eight sound-attenuating chambers and a computer control unit attached to a standard desktop computer operating Windows NT. The testing restraints within each startle chamber were made of clear Plexiglas and were 3.81×8.89 cm with an adjustable ceiling set to 2.2 cm. Movements within the cylinder were transduced by a piezoelectric disk attached to the Plexiglas base. The platforms were calibrated daily for accuracy and were adjusted to 1000 ± 10 mN. Between subjects and sessions the platforms were cleaned with Skilcraft Clean (Lighthouse of Houston, Houston, TX, USA). Acoustic stimuli were presented through a loudspeaker mounted 24.45 cm above the animal. A modified Realistic sound level meter (Hamilton Kinder, Poway, CA, USA), with the microphone placed in the location of the subject's head, verified the sound pressure level (SPL).

2.5. Acoustic stimuli

A 60-dB SPL, full-spectrum, 2–40 kHz white noise was presented as a continuous background stimulus in each startle chamber. In total, six trial types were employed: 120-dB noise bursts alone, 120-dB noise bursts with a prepulse, 100-dB noise bursts alone, 100-dB noise bursts with a prepulse, 70-dB prepulse-alone trials and no stimulus (60 dB background alone). Each trial consisted of 15 ± 5 s of background noise that culminated with a presentation of 50 ms (1–2 ms rise/fall time), 60-, 70-, 100- or 120-dB, white noise test stimulus. The 60- and 70-dB stimuli were stimulus control conditions presented to ensure that there was not significant activity within the recording chamber during testing and to ensure that the 70-dB stimulus alone did not elicit a startle reflex. The 100- and 120-dB stimuli served as startle-eliciting stimuli. For PPI trials, a 70-dB, 50 ms white noise (i.e., prepulse) preceded the 100- or 120-dB startle-eliciting stimulus by 50 ms.

2.6. Behavioral procedure

Acoustic startle responses were measured during the light phase of the light/dark cycle and subjects were tested only one session per day. Mice were placed in the Plexiglas restraint in the dark sound-attenuating chamber. Each test session began with a 3-min chamber adaptation period during which only the background stimulus was presented. The six stimulus trials were presented ten times each using a randomized block design totaling 60 trials. Inter-trial intervals were 15 ± 5 s. Each animal's movement was measured for a period of 200 ms following onset of the test stimulus. The peak startle amplitude (V_{max}) was recorded as the highest observed force occurring during the 200-ms assessment period. The time to peak startle amplitude (T_{max}) was the time the V_{max} occurred following test stimulus onset. The amount of prepulse inhibition (PPI) produced was calculated following behavioral testing and equaled the difference in startle magnitude between the pulse-alone and the prepulse plus pulse trials, divided by the startle magnitude for the pulse-alone trials, multiplied by 100.

Subjects were placed in the startle chambers and acclimated to the apparatus one session per day for six days prior to drug testing. The last two sessions, the mice were exposed to a subcutaneous (sc) and intraperitoneal (ip) injection of 200 μl saline that mimicked the procedure and timing employed during the drug assessment (see below). A combined mean V_{max} to 100- and 120-dB noise bursts was computed across the last three pretest (baseline) sessions for each subject and the 40 subjects were matched and divided into four equal experimental groups (saline–saline, saline–physostigmine, HuBChE–physostigmine, and HuBChE–saline) using this startle measure. Two hours before the next test session, the subjects were pretreated with saline or HuBChE (2000 U, ip). Pilot testing revealed that butyrylcholinesterase activity peaked between 2 and 4 h after ip administration. Because a session lasted for approximately 15 min, 2 h was selected as the pretreatment time prior to behavioral assessment so that it would approximate the time of maximum circulating cholinesterase activity. Fifteen minutes before testing, subjects were exposed to either saline or physostigmine hemisulfate (0.4 mg/kg, sc). As reviewed by Triggle et al. (1991), plasma physostigmine concentrations peak 5 min following im administration and brain concentrations peak 22 min following oral administration in the rat. Based on this information, startle testing began 15 min post sc administration. A pilot assessment confirmed that the physostigmine dose and administration time employed would alter startle behavior (unpublished results).

2.7. Statistical analysis

Means for the drug assessment and analysis were computed for each variable (V_{max}, T_{max} and PPI) for each animal and aggregated for each treatment group (saline–saline, saline–physostigmine, HuBChE–physostigmine, and HuBChE–saline) and each session. V_{max}, T_{max} and PPI data for the three baseline sessions that preceded the drug assessment session were compared using a repeated measures analysis of variance (ANOVA) to confirm that the groups did not differ. Group (saline–saline, saline–physostigmine, HuBChE–physostigmine, and HuBChE–saline) served as a between subjects independent variable and Session (1–3) as a within subjects independent variable. Where necessary, significant baseline differences were further explored using a Scheffé post-hoc comparison.

Data for the drug assessment session were analyzed using planned comparisons because this experiment was specifically designed to address the predictions indicated in the introduction. The predictions were that physostigmine altered startle behavior, HuBChE did not (and thus was safe as measured by this behavior), and that HuBChE protected against the deleterious effects of physostigmine.

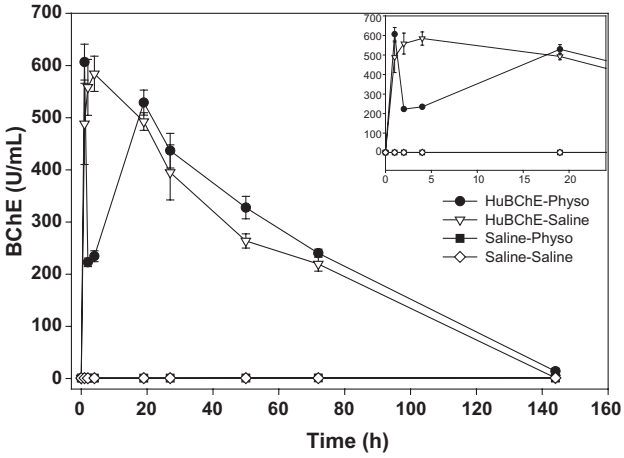

Fig. 1. Mean ± SEM blood BChE activity following HuBChE exposure and the saline or physostigmine challenge. The four experimental groups were saline–saline ($n=5$), saline–physostigmine ($n=5$), HuBChE–saline ($n=3$), and HuBChE–physostigmine ($n=8$). Inset is the first 24 h.

To that end, normally distributed startle data were analyzed using four planned comparisons to determine significant mean differences between groups. A Bonferroni adjustment was made to maintain the family wise error rate (α) of 0.05. The adjusted α for each of the four comparisons was therefore 0.0125. To assess the impairment caused by physostigmine, the saline–saline group was compared against the saline–physostigmine group. To provide a behavioral safety assessment of HuBChE, the saline–saline group was compared to the HuBChE–saline group. To assess the protection against physostigmine afforded by HuBChE, the HuBChE–physostigmine group was compared against both the HuBChE–saline and saline–physostigmine groups, respectively. These above four comparisons were completed for each dependent variable, V_{max}, T_{max} and PPI, using the 120-dB startle-eliciting stimulus.

3. Results

3.1. Circulating BChE time-course

Blood BChE activity before and after injection of HuBChE, saline, and the physostigmine challenge is shown in Fig. 1. A rapid increase in BChE following HuBChE administration (2000 U) reached peak levels between 2 and 4 h. For groups that received HuBChE, the observed peak levels of the enzyme were approximately 600 U/ml. For groups not exposed to HuBChE, blood BChE activity was constant at approximately 1 U/ml for the duration of the sampling period. In the HuBChE–physostigmine group, there was a dramatic reduction in circulating BChE levels following the physostigmine challenge, 2 h after the initial administration of HuBChE. At the 19-h serial time point, BChE levels were comparable for the two groups administered HuBChE.

3.2. Peak startle amplitude (V_{max})

Activity during the presentation of the 60- and 70-dB stimuli was roughly equal for all groups around 0.01 N (near the limit of reliable sensitivity for this apparatus) for these stimuli and these data are not presented. Given the similarity of the results using 100- and 120-dB startle-eliciting stimuli, data and statistical analyses are only presented for the more commonly employed and accepted 120-dB startle-eliciting stimulus. Fig. 2A shows the mean V_{max} for the 120-dB stimulus for the four groups. The repeated measures ANOVA confirmed the four treatment groups did not differ prior to drug assessment (Group $F_{3,36}<1$, ns; Session $F_{2,72}<1$, ns; Interaction $F_{6,72}<1$, ns). For the safety assessment, no significant difference ($F_{1,36}=4.32$, ns) was evident between the saline–saline group and the HuBChE–saline group. The positive control assessment compared the saline–saline group against the saline–physostigmine group and indicated that the V_{max} was significantly lower in the

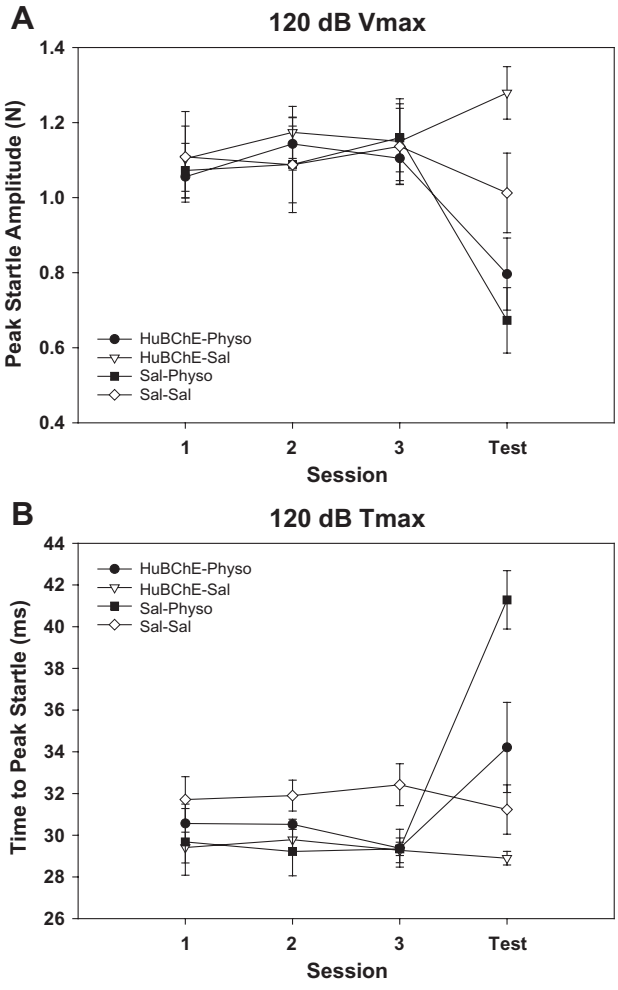

Fig. 2. (A) Mean ± SEM peak startle amplitude (V_{max}) in Newtons of force during three baseline sessions and the fourth test session. (B) Mean ± SEM time to peak startle amplitude (T_{max}) in milliseconds. Sessions 1–3 demonstrate the pre-exposure baseline behavior. Session 4 is the test session. Each group included 10 adult male C57BL/6J mice ($N=40$).

group that was exposed to physostigmine ($F_{1,36}=7.00$, $p<.01$). The protection comparison of the HuBChE–physostigmine group against the saline–physostigmine group revealed no group difference ($F_{1,36}<1$, ns). Further, the HuBChE–physostigmine group exhibited a significantly smaller peak startle amplitude than the HuBChE–saline group ($F_{1,36}=14.16$, $p<.01$). HuBChE did not prevent physostigmine-induced alterations in the amplitude of the acoustic startle response.

3.3. Time to peak startle amplitude (T_{max})

Fig. 2B shows the mean time to peak startle amplitude for the four groups of subjects for three baseline sessions and the test session. For T_{max}, the repeated measures baseline ANOVA indicated a significant main effect for Group ($F_{3,36}=3.60$, $p<.05$). The Scheffé post-hoc analysis did not indicate a significant difference between groups during the baseline period. The four treatment groups did not exhibit an effect of Session ($F_{2,72}<1$, ns) or a significant interaction ($F_{6,72}<1$, ns) prior to drug assessment. For the safety assessment, there was not a significant difference ($F_{1,36}=1.34$, *ns*) between the saline–saline group and the HuBChE–saline group on T_{max}. The control assessment comparing the saline–saline group against the saline–physostigmine group indicated the time to peak startle amplitude was significantly ($F_{1,36}=24.82$, $p<.01$) delayed in subjects that were exposed to physostigmine. The protection comparison of the HuBChE–physostigmine group against the saline–physostigmine group showed that the group not receiving HuBChE exhibited a significantly longer T_{max} ($F_{1,36}=12.30$, $p<.01$) indicating protection against the detrimental effects of physostigmine. However, the T_{max} was significantly longer ($F_{1,36}=6.93$, $p=.0124$) for the HuBChE–physostigmine group than for the HuBChE–saline group, indicative of only partial protection against the behavior-altering effects of physostigmine.

3.4. Prepulse inhibition (PPI)

Fig. 3 shows the mean prepulse inhibition of the four groups of subjects. The repeated measures ANOVA confirmed the four treatment groups did not differ prior to drug assessment (Group $F_{3,36}<1$, ns). There was, however, a main effect of Session ($F_{2,72}=4.34$, $p<.05$) but no significant interaction ($F_{6,72}<1$, ns). The Scheffé post-hoc revealed that behavior during Session 2 significantly differed from Session 1, but not Session 3 collapsed across groups. Given no differences between baseline Sessions 1 and 3, this result is difficult to interpret. For the safety assessment, there was not a significant difference ($F_{1,36}=3.94$, ns) between the saline–saline group and the HuBChE–saline group. The control assessment comparing the saline–saline group against the saline–physostigmine group demonstrated that PPI was significantly greater ($F_{1,36}=39.41$, $p<.01$) in the subjects exposed to physostigmine. The protection comparison of the HuBChE–physostigmine group against the saline–physostigmine group showed that the group receiving HuBChE exhibited significantly less PPI ($F_{1,36}=16.89$, $p<.01$) indicating protection against the deleterious effects of physostigmine. However, significantly greater ($F_{1,36}=17.25$, $p<.01$) PPI was observed for the HuBChE–physostigmine group relative to the HuBChE–saline group. Again, these latter assessments demonstrated that HuBChE provided only partial protection against the increased PPI resulting from physostigmine exposure.

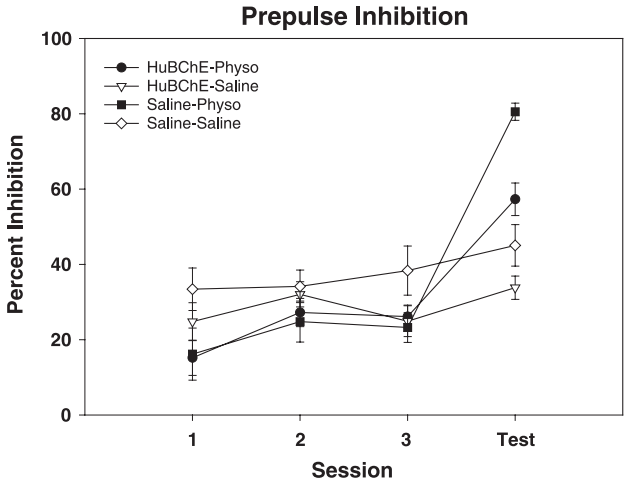

Fig. 3. Mean±SEM prepulse inhibition (PPI). Sessions 1–3 demonstrate the pre-exposure baseline behavior. Session 4 was the exposure test session. PPI is calculated as the difference between the behavioral response to the 120-dB pulse-alone and the response to the 70-dB prepulse and 120-dB pulse stimulus pair divided by the response to the 120-dB pulse-alone, multiplied by 100.

4. Discussion

We assessed the time-course of HuBChE in the circulation of naïve male C57BL/6J mice and demonstrated residence times similar to those observed in rats (Genovese and Doctor, 1995), guinea pigs (Allon et al., 1998), rhesus monkeys (Raveh et al., 1997), and other studies including mice (Raveh et al., 1989; Saxena et al., 1997). Collectively, these results demonstrate the relative stability of exogenously administered heterologous BChE in various species. The residence time of HuBChE in humans will likely be more stable and sustained over a longer timeframe. This conclusion is supported by the research of Rosenberg et al. (2002) showing enzyme residence time following administration of homologous BChE (i.e., from other nonhuman primates) over a period of days. This further supports research in BChE "deficient" humans that were exogenously administered HuBChE where the stability half-life was approximately two to 12 days (Garry et al., 1974; Jenkins et al., 1967; Ostergaard et al., 1988; Schuh, 1977; Stovner and Stadskleiv, 1976). Subjects that received both

HuBChE and the physostigmine challenge exhibited approximately 60% of the enzyme activity present in the unchallenged group. This result is consistent with a 1.6 molar ratio of HuBChE to physostigmine and shows that stoichiometrically there was sufficient enzyme to neutralize the dose of physostigmine.

Behaviorally, the effects of HuBChE and physostigmine on the acoustic startle reflex, the time to peak startle amplitude, and prepulse inhibition were examined in the current study. As expected, physostigmine, a short-acting, efficient, and effective carbamate ChE inhibitor (Triggle et al., 1998), significantly decreased the amplitude of the acoustic startle reflex. Further, the time to the peak startle reflex was significantly increased, as was the amount of PPI of the acoustic startle reflex, because of exposure to physostigmine. These results support and extend the previous work that showed a significant alteration in startle reactivity following exposure to OP and carbamate inhibitors (Haggerty et al., 1986; Jones and Shannon, 2000b; Philippens et al., 1997; Philippens et al., 1996; Waite and Thal, 1995).

These are some of the first data to demonstrate alterations in PPI due to acute physostigmine exposure. Previously, Jones and Shannon (2000b) exposed rats to two reversible cholinesterase inhibitors, tacrine (0.3–10 mg/kg) and physostigmine (0.01–0.1 mg/kg), without observable alterations in PPI behavior. The current study employed a 0.4 mg/kg dose of physostigmine and there was a very apparent increase in PPI. This suggests that a more complete dose–response experiment could explore the effect of physostigmine on PPI. Alternatively, the current experiment suggests that excessive HuBChE does not alter PPI. Thus, the question remains in general whether the alteration in PPI is due to cholinesterase inhibition, or to an alternate drug action. Because there is a paucity of experiments examining the impact of cholinesterase modulation on PPI overall (Geyer et al., 2001), a direct examination of PPI and cholinesterase modulation is required to fully characterize the role of this neurochemical.

Despite the consistent results across mice and rats showing decreased startle reactivity resulting from cholinesterase inhibition, there is a caveat regarding this observation. Rats, mice and guinea pigs exposed to ChE inhibitors have repeatedly exhibited altered startle reactivity, but the direction of the effect appears to be species dependent. The current research and research with rats has indicated depressed and delayed startle reactivity following ChE inhibition (Haggerty et al., 1986; Jones and Shannon, 2000b; Waite and Thal, 1995). Conversely, guinea pigs showed an *enhanced* startle reactivity, including both a shorter latency and increased amplitude, following physostigmine administration (Philippens et al., 1997; Philippens et al., 1996). There is currently no data regarding the reason for this species difference. An understanding of this difference between mice, rats, and guinea pigs requires direct empirical evaluation and may provide a better understanding of the role of the cholinergic system in acoustic startle behavior.

When physostigmine was presented as a pharmacological challenge to subjects that received HuBChE, some protection was provided against the behavior-altering effects of physostigmine. The question remains why the HuBChE protection was incomplete despite the excess stoichiometric ratio of HuBChE to physostigmine. While not clear, it is possible that the observed partial protection to physostigmine is due to the pharmacokinetics of this drug (e.g., ChE binding, carbamylation and decarbamylation). For example, Somani and Dube (1989) examined the dose–response of physostigmine (25–500 μg/kg) on red blood cell and brain ChE inhibition in rats. In blood, they observed that cholinesterase inhibition was maximal at 40% with a dose of 200 μg/kg. Blood ChE inhibition did not increase for exposures in excess of this amount, despite sufficient circulating ChE. However, the level of brain ChE inhibition continued to rise linearly and was maximal at the highest exposure of 400 μg/kg. These data indicate that because of the bimolecular rate constant, it is possible that the physostigmine is not sequestered and the remaining inhibitor could partition into the CNS where it could produce its deleterious effect.

The primary purpose of this research was to examine the behavioral toxicity of purified HuBChE in a startle assessment. With regard to this, no significant changes in startle behavior were observed following exposure to HuBChE alone. This observation is particularly compelling when compared with the results of the circulatory stability assessment. In that assessment, subjects receiving exogenously administered HuBChE showed a mean circulating BChE level approximately 600× higher than the normal physiological levels (see Fig. 1). Moreover, a dose of 2000 U of HuBChE is a very large dose for a 30 g mouse and is equivalent to 30× the estimated human dose necessary for protection against 2 LD_{50} of soman. This result supports the work of others indicating the lack of a behavioral side effect for BChE. Genovese and Doctor (1995) exposed rats to EqBChE and tested them in a battery of behavioral tasks including passive avoidance, locomotor activity, and standard schedule-controlled behavior. No significant alteration in behavior was observed in these tasks following exposure to EqBChE. Matzke et al. (1999) exposed rhesus monkeys to EqBChE and failed to observe any decrement in performance on a six-item serial probe recognition task following two separate administrations. The current results also support the observation by Raveh et al. (1997) of no observable deficits on a spatial discrimination task resulting from HuBChE administration alone in rhesus macaques. Raveh et al. employed a maximum dose roughly equivalent to 11 mg/kg (9000 U/kg). In the current experiment, the mice received a dose of roughly 95 mg/kg (66,700 U/kg), and still exhibited no apparent behavioral toxicity to HuBChE. Thus, even high doses of HuBChE appear behaviorally safe.

These results clearly indicate that a large exogenous administration of HuBChE has no observable effect on startle behavior. Further research is underway to reexamine and confirm the impact (or lack thereof) of a comparable dose of HuBChE on a more complex neurobehavioral learning and memory assessment with mice and nonhuman primates. The current research contributes to the expanding body of work in various species indicating that HuBChE is a safe and effective bioscavenger that should be developed for future inclusion in the protective regimen against OP chemical warfare nerve agents.

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