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The Effects of Acute and Repeated Pyridostigmine Bromide Administration on Response Acquisition with Immediate and Delayed Reinforcement

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VAN HAAREN, F., R. DE JONGH, J. B. HOY, J. L. KARLIX, C. J. SCHMIDT, I. R. TEBBETT AND D. WIELBO. *The effects of acute and repeated pyridostigmine bromide administration on response acquisition with immediate and delayed reinforcement.* PHARMACOL BIOCHEM BEHAV **62**(2) 389–394, 1999.—This experiment was designed to assess the effects of acute and repeated administration of pyridostigmine bromide (a carbamate with prophylactic and therapeutic uses) on response acquisition. Experimentally naïve, male Sprague–Dawley rats were exposed to a situation in which lever presses were either immediately followed by food-pellet presentation or after a 16-s resetting delay. Different groups of rats received either one acute administration of pyridostigmine bromide (10 mg/kg, by gavage) or repeated pyridostigmine administration for 7 days (1.5 mg/kg/day, by gavage). Other groups were treated with distilled water for the same period of time. Both acute and repeated pyridostigmine bromide administration decreased serum cholinesterase levels by approximately 50%, but neither treatment affected brain cholinesterase levels in our assay. Acute and repeated drug administration produced the same behavioral effects. Subjects exposed to the 0-s delay conditions obtained many more food pellets than those exposed to the 16-s delay conditions. Administration of pyridostigmine bromide delayed the onset of responding in some, but not all, of the subjects in the treated groups, independent of the delay condition to which they were exposed. Many more responses were observed on an inoperative lever during the 16-s delay conditions than during the 0-s delay conditions, especially during the 16-s delay condition in which subjects had received acute vehicle administration. Whether or not these effects of small doses of pyridostigmine bromide on response acquisition are of central or peripheral origin will need to be determined in future studies, as response acquisition in the present experiment may have been affected by pyridostigmine's effects on gastrointestinal functioning and/or motor activity. © 1999 Elsevier Science Inc.

Gulf War Syndrome Pyridostigmine bromide Cholinesterase inhibition Response acquisition Delayed reinforcemen Lever press Male rats

PYRIDOSTIGMINE bromide (PB), a quaternary carbamate, is a reversible inhibitor of acetylcholinesterase (AchE), thereby causing acetylcholine (Ach) to accumulate at receptor sites (18). PB is used in the treatment of myasthenia gravis (7), and as pretreatment under threat of chemical warfare because of its protective effect against organophosphorus (OP) nerve gases (3,5). OP agents exert their effect by irreversibly inactivating AchE resulting in signs and symptoms consistent with excess cholinergic stimulation. PB protects against OP poisoning by shielding AchE through reversible inhibition of the enzyme in the peripheral nervous system [cf. (1,2)]. Spontaneous decarbamylation occurs following treatment with PB

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restoring the activity of AchE (20). PB was taken prophylactically by an estimated 250,000 soldiers during the Gulf War.

Evidence has been presented to show that small amounts of PB are behaviorally active after acute administration. Wolthuis and Vanwersch (21) reported in 1984 that intraperitoneally (IP) administered PB interfered with two-way shuttlebox-avoidance learning, open-field behavior, and complex coordinated movements in rats, without producing overt symptoms and without affecting running speed and simple coordinated locomotion. Similarly, Shih et al. (15) found that low doses (6 and 12 mg/kg) of orally administered PB produced a decrement in operant responding maintained under a multiple fixed-ratio, time-out (multi-FR-TO) schedule of water reinforcement. Consistent with these results is a study by Liu (12), who showed that low doses (3–12 mg/kg) of orally administered PB dose dependently decreased the rate of responding for water reinforcement in a visual intensity discrimination task, again without producing signs of overt toxicity. PB also dose dependently decreased unconditioned water intake in water-deprived rats, but did not significantly affect locomotor activity. On the basis of these results, the author suggested that the disruptive effects of PB on the performance in the simple light intensity discrimination task involved motivational dysfunction rather than motor impairment. However, Hoy et al. (8) have recently presented evidence to show that acute PB administration in the range of that investigated by Liu (12) dose dependently decreased spontaneous locomotor activity in male and, even more so, in female Sprague–Dawley rats.

The present experiment is one of several designed to assess the effects of repeated PB administration on the acquisition of a novel response (learning) in rats. Previous experiments have shown that food-deprived, but magazine-trained, rats will quickly learn to contact a lever in an operant chamber. They will continue to contact the lever at high rates when lever contacts are followed by food presentation (11,19). This paradigm has proven useful to assess the effects of a pharmacological challenge on the acquisition of a new response, thereby providing important information on response acquisition that cannot be derived from assessing drug effects on well-established performance. For instance, Stolerman (16,17) has reported that chlorpromazine and chlordiazepoxide impaired response acquisition when lever presses were immediately followed by pellet presentation. More recently, LeSage et al. (11) have presented evidence to show that rats learn to press a lever following *d*-amphetamine (*d*-AMPH) administration both when pellet presentation occurs immediately following the response or after the expiration of a resetting delay. Differential responding on the operative lever (an index of acquisition) was not affected by *d*-AMPH, however, which led the authors to conclude that this compound did not disrupt response acquisition, except at doses that produced a general disruption in behavior.

The present experiment was designed to assess the effects of acute and repeated PB administration on the acquisition of a lever press response when lever presses were either immediately followed by pellet presentation (delay 0-s) or after the expiration of a 16-s resetting delay (resetting delay 16-s). Previous studies have suggested that the detrimental behavioral effects of drugs or toxins may be more easily recognized under the latter conditions (11). Adult male rats either received one acute administration of a small dose of PB or they were treated with PB for 7 days prior to the acquisition session. The latter treatment conditions (1.5 mg/kg/day) approximated those of the Gulf War, during which soldiers sometimes were ordered to take 3 × 30 mg PB/day/70 kg for 1 or 2 weeks (9).

METHOD

Subjects

Forty-eight experimentally naïve male Sprague–Dawley rats were obtained from a commercial supplier (Harlan–Sprague–Dawley, Indianapolis, IN) when they weighted between 250–275 g. They were housed in groups of three under a reversed 12-h light–dark cycle (lights on 1800 h), in a temperature- and humidity-controlled environment. The rats were handled daily for 2 weeks before the beginning of the experiment. Standard rodent chow was available in the home cages during the first week. Starting with the second week, home cage rodent chow was limited to approximately 16 g per rat per day, delivered at approximately 1600 h. Water was continuously available in the home cage.

Apparatus

The experiments were conducted in six rodent operant conditioning chambers (Coulbourn Instruments, Allentown, PA). The chambers were 25 cm wide, 30 cm long, and 29 cm high. The side walls were made of Plexiglas and the intelligence panel and the back wall consisted of modular stainless steel panels. The floor consisted of 16 rods, spaced 1.75 cm apart. A pellet tray was located 1.7 cm above the floor in the middle of the intelligence panel, and a houselight was approximately 3 cm from the ceiling of the chamber. The pellet tray could be illuminated during pellet presentation (Noyes, 45 mg rodent purified formula). There were two retractable levers, one to the right and one to the left of the pellet tray. They were spaced 12.5 cm apart and located 6.3 cm above the floor. The levers protruded 1.8 cm from the intelligence panel. Each chamber was enclosed in a sound-attenuating and ventilated cubicle. Experimental events were controlled and data were collected using an IBM compatible computer (GatorByte, Gainesville, FL) with L2T2 software and LabLinc interfacing obtained from Coulbourn Instruments (Allentown, PA).

Procedure

Groups of six rats were exposed to one of eight different experimental conditions. The delay of reinforcement was either 0 s (delay 0-s) or 16 s (delay 16-s resetting). The drugs were administered either acutely or repeatedly, and the rats received either PB or distilled water (PB vehicle). When the drugs were administered acutely, the rats were first trained to retrieve food pellets from the tray in the operant chamber (magazine training). During magazine training, the rats were first placed in the darkened operant chamber and both levers were retracted from the chamber. After 5 min, the houselight was illuminated and pellets were delivered on a variable time (VT) 60-s schedule. Both levers remained retracted during the magazine training session, which was terminated after 60 pellets had been delivered. Subsequently, the rats received distilled water by gavage for 2 days. They were tested 30 min following PB or vehicle administration on day 3. When the drugs were administered repeatedly, the rats were also first trained to eat from the pellet tray. Then, for 7 days, they received either PB or distilled water by gavage, and they were tested 30 min after drug or vehicle administration on day 7.

The acquisition session (which started at 1600 h to include the final 2 h of the subject's dark period) also began with a 5-min dark period, during which the levers were retracted from the chamber. Then, the houselight was illuminated and both levers were extended into the operant chamber. Pressing the left (operative) lever immediately resulted in pellet pre-

sentation during the 0-s delay condition. In the delay condition, pressing the left lever resulted in pellet presentation after 16 s, but only if the subject did not press the lever during the (unsignaled) delay interval. A press on the left lever during the delay reinitiated the delay interval. In both conditions, pressing the right (inoperative) lever had no scheduled consequences. The experimental session was terminated after 8 h and the rats were removed from the experimental chamber and returned to the home cage at that time. The data for the different groups of subjects were collected on consecutive days.

Drugs

Pyridostigmine bromide (PB, Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water, and both PB and distilled water were administered by gavage, in a volume of 5 ml/kg. PB was either administered at 10 mg/kg, 30 min prior to the beginning of the experimental session (acute administration) or at 1.5 mg/kg for 7 days at approximately 30 min prior to the scheduled starting time of the experimental session on day 7 (repeated administration).

Serum Preparation

Trunk blood was collected from the six PB-treated rats and the six control rats that participated in the repeated-administration experiment. They received one more administration of PB or vehicle on the day after the response acquisition session 30 min prior to blood collection. The trunk blood of rats who received one acute administration of PB or distilled water was obtained from a group of subjects that had not participated in the response acquisition session, but that, otherwise, had been treated in a manner identical to that of the subjects who participated in the experiment. To collect blood, the rat was placed in a jar containing a paper towel saturated with Metofane (Methoxyflurane), 30 min after PB or vehicle administration. The anesthetized animal was quickly decapitated after 1 min. Blood was collected in a 15-ml polystyrene culture test tube and allowed to coagulate on ice for 2 h. It was then centrifuged for 15–20 min at approximately 3000 revolutions per minute. The serum was then drawn off the solid cell matter with a clean glass Pasteur pipette and placed in a 1.5-ml polystyrene microcentrifuge tube. It was then immediately placed in a freezer (at −20°C) where it was stored for up to 3 months until analysis. Brains were also removed at the time of decapitation, quickly frozen, and stored in the freezer.

Serum Analyses

Pyridostigmine bromide. The serum sample (0.5 ml) was transferred to a stoppered tube and vortexed with 1 ml of 0.025 M potassium phosphate buffer at pH 3. This mixture was then applied to a Strong Cation Exchange column that had previously been conditioned under vacuum on a Vac Elut manifold (Analytichem) with methanol (2 ml), water (1 ml), and 0.25 M phosphate buffer (1 ml). After application of the sample, the column was air dried for approximately 30 s and then washed with phosphate buffer (1 ml) and 0.1 M acetic acid. The column was again air dried for 30 s before eluting off the adsorbed drugs with ammoniacal methanol (3%, 2 ml). The final extract was evaporated to dryness under nitrogen and the residue reconstituted in 50 μl of methanol. A 20-μl aliquot of the extract was used for HPLC analysis. This analysis was performed using a Waters 510 pump to deliver solvent at 1 ml/min to a Hypersil 5 μm ODS (25 cm × 4.5 mm i.d.) column. A Waters C18 Guard Pak precolumn was used to protect the analytical column. The Detector was a Waters 486 variable wavelength detector set at 272 nm with a Dell 486 data system and Millenium™ software. The mobile phase consisted of acetonitrile–0.1% triethylamine in water (adjusted to pH 3.2 with phosphoric acid 70:30). Quantitative analysis was achieved by comparison of peak areas with unextracted standards. Each determination was taken as the mean of three replicate injections. The calibration graph was produced over the range of 0.05–5 μg/ml. The sensitivity of the assay was 0.05 μg/ml.

Serum cholinesterase. Prepared test kits (Sigma, St. Louis MO, 420-MC) were used to measure cholinesterase activity. This assay is based on the method of Rappaport et al (14), and depends on the quantitative formation of acetic acid from acetylcholine in the presence of an acid-based indicator, *m*-nitrophenol. All assays were done in triplicate.

Brain cholinesterase. Half a brain (approximately 0.9 g) was placed in a 15-ml conical polypropylene tube with 5 ml of Dulbecco's phosphate-buffered salt solution. The tissue was homogenized in a Tissue Tearor (model 985-370) for about 2 min. Tubes were then capped and centrifuged at 4000 rpm for 20 min at 4°C. The supernatant was then assayed as described above.

RESULTS

Serum samples were analyzed for the presence of PB and the extent of cholinesterase inhibition following PB administration. Acute administration of 10 mg/kg PB resulted in serum levels that averaged 175 ng/ml, ± 32.42 ng/ml (SEM). PB could not be detected in three of the six serum samples obtained 30 min following the final administration of 1.5 mg/kg PB, but PB averaged 83 ng/ml, ± 7.23 ng/ml (SEM) in the serum of the remaining three subjects. Acute administration of 10 mg/kg PB resulted in a 57% decrease in serum cholinesterase levels compared to vehicle administration, $t(10) = 3.11$, $p < 0.01$. Similarly, repeated administration of 1.5 mg/kg/day for 7 days decreased serum cholinesterase activity compared to vehicle administration by about 47%, $t(9) = 2.53$, $p < 0.03$. Acute or repeated PB administration did not affect brain cholinesterase levels.

Figures 1 and 2 show the cumulative number of reinforced responses on the operative lever for individual subjects during the 0-s delay condition (Fig. 1) and the 16-s delay condition (Fig. 2) after acute and repeated vehicle administration (left panels) and after acute and repeated PB administration (right panels). The open circles connected by the solid lines represent group-averaged cumulative responses on the inoperative lever. Note the difference in the vertical axes between Figs. 1 and 2.

The data shown in Figs. 1 and 2 suggest that both delay duration and PB administration affected the number of responses on the operative lever. Note that some subjects failed to acquire the operant response altogether, especially following acute PB administration in the 16-s delay condition. Responses on the operative lever were analyzed by ANOVA, which included the between-subject variables delay (0 s or 16 s), treatment (acute or repeated), and drug (PB or vehicle) and the within-subject variable time (cumulative number of responses observed at each full hour of the experimental session). A number of relevant observations may be described. First of all, subjects exposed to the 0-s delay condition obtained more food pellets than those exposed to the 16-s resetting delay condition [delay: $F(1, 39) = 54.81$, $p < 0.0001$]. Secondly, all subjects obtained more food pellets as the session progressed [time: $F(7, 280) = 31.95$, $p < 0.001$]. There were no

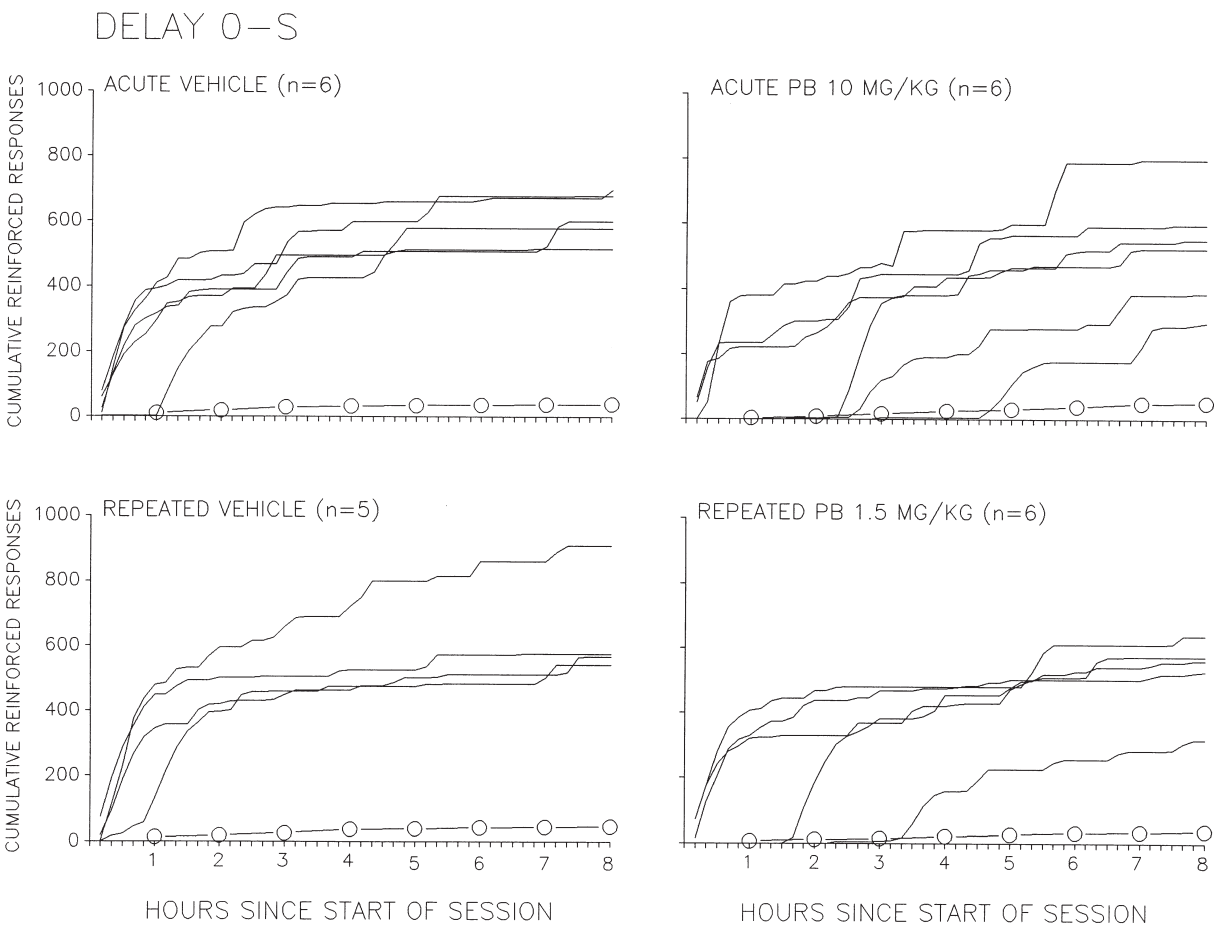

FIG. 1. The cumulative number of reinforced responses for individual subjects during the 0-s delay condition after acute or repeated vehicle administration (left-hand panels) and following the acute administration of 10 mg/kg PB or the repeated administration of 1.5 mg/kg PB for 7 consecutive days (right-hand panels). The open circles connected by the solid lines represent group-averaged cumulative responses on the inoperative lever.

differences between groups as a function of acute or repeated drug administration [treatment: $F(1, 39) = 0.11$, NS]. PB did not appear to affect the number of obtained food pellets [drug: $F(1, 39) = 2.85$, $p < 0.099$], but a significant three-way interaction involving the variables delay, drug, and time, $F(7, 280) = 2.98$, $p < 0.0050$, suggested drug involvement. It can be seen in Figs. 1 and 2 that 1) subjects were more likely to earn food pellets during the earlier parts of the session during the 0-s delay condition than during the 16-s delay condition [delay × time: $F(7, 280) = 10.24$, $p < 0.0001$]; 2) that PB administration delayed the onset of responding in some, but not all of the subjects in the drug-treated groups [drug × time: $F(7, 280) = 1.88$, $p < 0.0734$, NS]; and 3) that the 0-s and 16-s delay conditions did not differentially affect the number of obtained food pellets following vehicle or PB administration [delay × drug: $F(1, 39) = 0.06$ NS]. ANOVA of the latencies until the first, fifth, and tenth reinforced response during the different experimental conditions (see Figs. 1 and 2) revealed a significant interaction between delay duration and drug treatment [delay × drug: $F(1, 39) = 4.21$, $p < 0.0468$], suggesting that latencies were longer in the 16-s delay condition [delay: $F(1, 39) = 3.48$, $p < 0.0696$] and following PB administration [drug: $F(1, 39) = 4.00$, $p < 0.0524$].

Figures 1 and 2 also reveal that the number of responses on the inoperative lever varied as a function of experimental conditions. ANOVA revealed that the number of responses on the inoperative lever was higher during the 16-s delay condition than during the 0-s delay condition [delay: $F(1, 39) = 7.57$, $p < 0.0090$] and that their number increased over time [time: $F(7, 280) = 17.98$, $p < 0.0001$], but more so during the 16-s delay condition than during the 0-s delay condition [delay × time: $F(7, 280) = 4.13$, $p < 0.0002$]. Many more inoperative responses were observed during vehicle than during PB administration [drug: $F(1, 39) = 4.76$, $p < 0.0352$], attributable mostly to a much higher number of responses on the inoperative lever during the acute administration of vehicle in the 16-s delay condition than in any of the other experimental conditions [delay × drug: $F(1, 39) = 3.94$, $p < 0.0541$, and treatment × drug: $F(1, 39) = 6.62$, $p < 0.0140$].

DISCUSSION

The results of this experiment confirm and extend observations from other studies. Experimentally naïve rats exposed to 0-s delay condition obtained many more food pellets than rats exposed to the 16-s resetting delay condition. As such, these

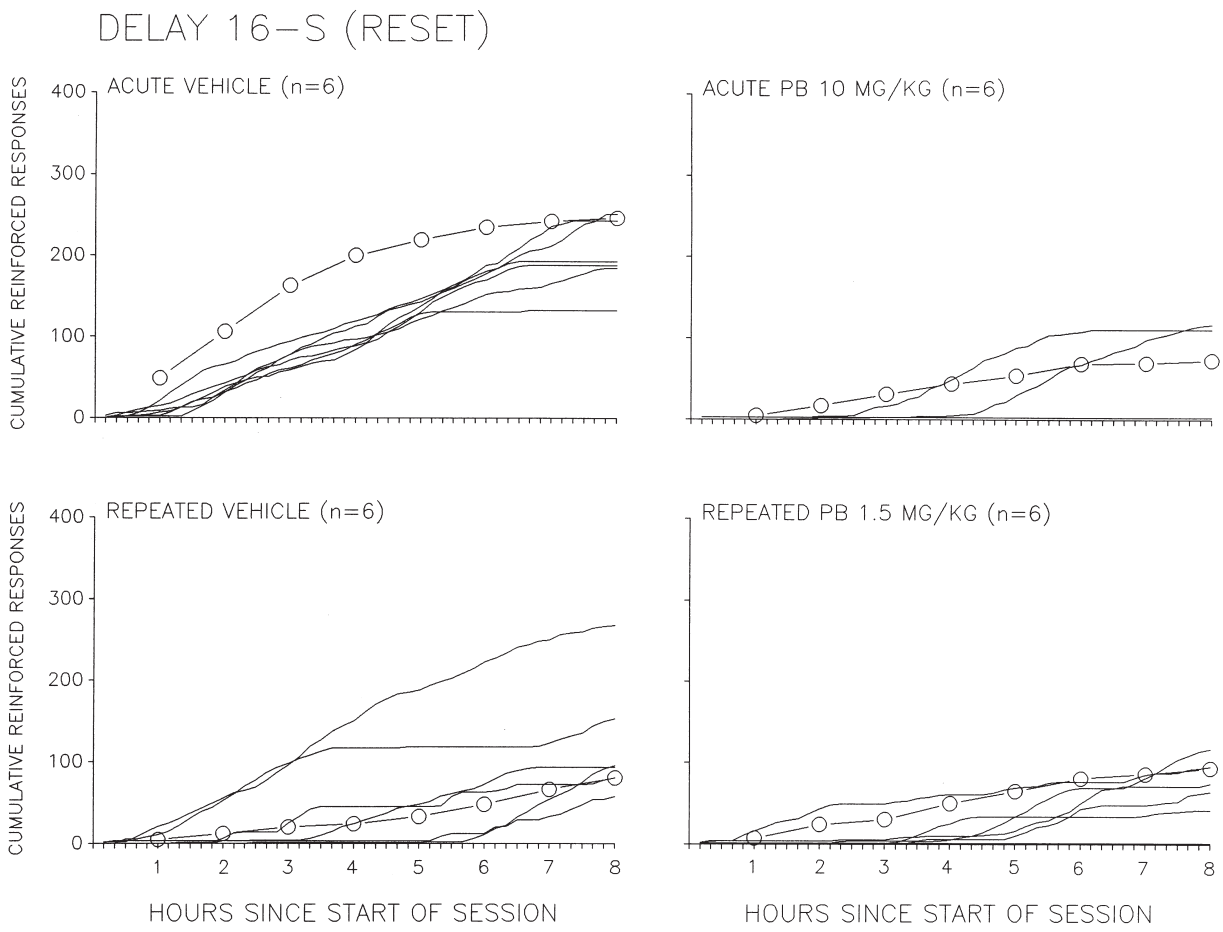

FIG. 2. The cumulative number of reinforced responses for individual subjects during the 16-s, resetting delay condition after acute or repeated vehicle administration (left-hand panels) and following the acute administration of 10 mg/kg PB or the repeated administration of 1.5 mg/kg PB for 7 consecutive days (right-hand panels). The open circles connected by the solid lines represent group-averaged cumulative responses on the inoperative lever.

results confirm those of other experiments in which it was shown that response-contingent delayed pellet presentation delays response acquisition (11,19). Acute administration of 10 mg/kg PB and repeated administration of 1.5 mg/kg/day for 7 days reduced cholinesterase activity by approximately 50%. There were no differences between groups as a function of acute or repeated PB administration, indicating that the cumulative effects of very small doses of PB (1.5 mg/kg/7 days) were similar to those of one much larger dose of PB (10 mg/kg). The data also showed that PB administration delayed the onset of responding in some, but not all, subjects in the PB-treated groups. These data imply that repeated administration of a very small dose of PB (1.5 mg/kg/day for 7 days) adversely affects response acquisition in experimentally naïve subjects. It should be noted that the repeated dose of PB was chosen to resemble that which was most commonly administered during the Gulf War, although Gulf War exposure may have been more prolonged (i.e., 3 × 30 mg/70 kg for 7–14 days). That particular treatment regimen has been stated to be safe and well tolerated in a double-blind evaluation of its safety, tolerance, pharmacokinetics, and pharmacodynamics in 90 male and female volunteers (10). These pharmacokinetic studies, however, did not assess any functional consequences of such drug administration regimen. The results of the present experiment appear to indicate that the functional consequences of this low dose of PB (lower than those that have been reported to facilitate drug interactions with such compounds as permethrin and DEET [cf. (1,2)] should not be underestimated.

An interesting question is whether PB causes these effects on behavior by acting on the central nervous system (CNS) or on the peripheral nervous system (PNS). It has been assumed that PB, as a quaternary carbamate, does not cross the blood–brain barrier (BBB). If that is true, it would seem that PB's behavioral effects should result from actions only on the PNS. However, there are a number of findings that indicate that PB's effects may be centrally mediated. First, PB at low doses that do not cause signs of toxicity, produced behavioral effects in paradigms that involve CNS activity (21). Secondly, pretreatment with PB protects against intoxication with soman, an OP nerve gas that predominantly acts in the CNS (3). Furthermore, disruption of the BBB might possibly allow PB administration to have central effects. Friedman et al. (4) showed in stressed mice that an increase in BBB permeability reduced the dose of PB required to inhibit brain AchE activity by 50% to less than $\frac{1}{100}$th of the dose required in non-

stressed mice. When PB was given to healthy volunteers during peacetime, only 8.3% of the subjects reported CNS symptoms (headaches, insomnia, drowsiness, nervousness, unfocused attention and impaired calculation capacities), whereas in soldiers treated during the Gulf War, 23.6% reported CNS symptoms, possibly due to enhanced stress levels under those conditions (4,6).

Although PB appears to have central effects, Liu (13) has argued that the detrimental effects of PB on operant behavior are mediated by peripheral muscarinic receptors. Liu studied the effects of atropine, a muscarinic antagonist with both a central and a peripheral action, and methylatropine, a muscarinic antagonist with only a peripheral action, on PB-induced (12 mg/kg) behavioral disruption during a brightness discrimination task. Atropine partially antagonized the PB-induced reinforcement loss, while at the same time increasing the number of nonreinforced responses. However, methylatropine completely antagonized the PB-induced reinforcement loss as well, without affecting the number of nonreinforced responses. This suggests that the detrimental effects of PB on operant behavior are due to the stimulation of peripheral muscarinic receptors, possibly in the gastrointestinal tract, because in humans, gastrointestinal disturbances are a common side effect of PB administration (18). Other studies conducted in our laboratories (8) have shown that acute PB administration at 10 mg/kg results in a sex-dependent decrease in locomotor activity in male and female Sprague–Dawley rats. This observation suggests that the effects of PB administration, at least in the acute conditions, may have produced effects on motor behavior that could have interfered with response acquisition as studied in the present experiment or the decrease may be symptomatic of the general malaise caused by PB. There are currently no data available with respect to the locomotor effects of repeated administration of very small doses of PB. The present experiment was not designed to evaluate these alternative explanations, but such experiments should be conducted in the future to arrive at a comprehensive understanding of the effects of acute and repeated PB administration on response acquisition. In particular, it might be worthwhile to determine PB effects on response acquisition in rats pretreated with methylatropine or methylscopolamine to block peripheral cholinergic muscarinic receptors.

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