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The effects of selective kappa-opioid, PD117302 ((±)-trans-N-mathyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzo[b]thiophene-4-acetamide), on transient (15min) global forebrain ischemia, induced by four-vessel occlusion, was evaluated using a multiple fixed-ratio, fixed-interval schedule of food presentation in rats. The schedule produced distinctive patterns of responding in the fixed-ratio and fixed-interval components. Ischemia produced CA1 hippocampal necrosis and prolonged suppression of responding under both schedule components. When responding resumed, the pattern of responding rapidly returned. Response disruption and CA1 hippocampal necrosis were minimal or nonexistant in sham-occluded rats. Behavioral recovery time under both components of the schedule of reinforcement correlated with CA1 necrosis. On average, CA1 necrosis was less, and behavioral recovery time was shorter in rats treated with PD117302 postocclusion as compared with vehicle-treated rats. The difference, however, did not reach statistical significance. These results demonstrate the utility of schedule-controlled responding for evaluating potentially theraputic compounds for the treatment of ischemic injury. These results also further characterize the neuroprotective actions of kappa opioids.

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Evaluation of Neuroprotection and Behavioral Recovery by the Kappa-Opioid, PD117302 Following Transient Forebrain Ischemia

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Ischemia Operant behavior Kappa opioid Rat

IN the rat, four-vessel occlusion (4-VO) (31) is a well-established procedure for the study of transient cerebral ischemia. Neuronal damage produced by 4-VO is noteworthy in that the CA1 field of the hippocampus is particularly vulnerable, although other brain areas, including the striatum and neocortex, are also vulnerable (29,31,32). Moreover, a substantial degree of necrosis is delayed for 24 h, or more, following the ischemic episode (26,32). Considerable evidence suggests that excessive glutamate- and aspartate-stimulated NMDA activity mediates delayed neuronal cell death [for reviews, see (3,23,33)].

Considerable effort, therefore, has been focussed on the search for compounds that may have therapeutic value in the treatment of ischemic injury. For example, competitive and noncompetitive NMDA antagonists have been shown to decrease neuronal loss following ischemic injury in rats and gerbils (9,10,35). Similarly, certain calcium channel blockers have been shown to improve outcome following 4-VO (13,30).

A number of selective kappa-opioid receptor agonists have also been shown to reduce ischemic injury. In the gerbil and rat, U-50,488 has been shown to protect against ischemia-induced lethality (36) and to reduce cerebral edema in the rat (34). U-50,488 and several of its analogs have also been shown to reduce ischemia-induced hippocampal cell loss in the gerbil (5,17). Similarly, GR89696 attenuates ischemia-induced CA1 hippocampal cell loss in the gerbil and mouse (1).

In the present study, we investigated the efficacy of the highly selective kappa-opioid receptor ligand, PD117302 (4,14–16,19–21,24,25), to reduce injury and improve behavioral outcome following ischemic trauma. PD117302 has been shown to reduce CA1 cell loss following ischemia in the gerbil (18). PD117302 has also been shown to protect against NMDA-induced convulsions in rats (38), and to reduce glutamate neurotoxicity in vitro (7). We evaluated behavioral outcome by measuring performance using a multiple schedule of food reinforcement. Schedule-controlled responding has been shown to be a valuable method for assessing a variety of pharmacological and physiological manipulations (e.g., 40). Additionally, we have previously reported that schedule-controlled responding is disrupted following brief 4-VO (12).

METHOD

Subjects and Apparatus

Experimentally and pharmacologically naive adult male Sprague–Dawley rats (Zivic–Miller, Pittsburgh, PA) were used. Rats were individually housed under a 12 h:12 h light:dark cycle

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(lights on at 0600), and water was always available in the home cages. Body weights were maintained at 325–350 g by food presented during experimental sessions and supplemental feedings occurring several hours after experimental sessions.

Experimental sessions were conducted in standard rodent operant conditioning chambers (model #E-10-10, Coulbourne Instruments, Lehigh Valley, PA), housed in ventilated, light- and sound-attenuating cubicles. Each chamber contained two response levers and a food trough that could be illuminated and was attached to a food dispenser capable of delivering 45 mg food pellets (Bio-Serv, Frenchtown, NJ). Each chamber also contained a houselight mounted on the ceiling and two stimulus lights mounted above each of the response levers. A response was considered to occur when either lever was pressed with a downward force of at least 0.3 N. Experimental events were controlled and monitored by a DEC, PDP-11/73 computer, using the SKED-11 software system.

Behavioral Procedure

Rats were trained to lever press for food pellets under a multiple fixed-ratio 20, timeout 15 s, fixed-interval 2 min, timeout 15 s, (FR20,FI2) schedule of reinforcement. Responses on only one lever produced food. Responses on the other lever were recorded but had no programmed consequences. Sessions consisted of repeating cycles of 3 FR20, 15 s timeout, FI2, 15 s timeout, and were approximately 60 min in duration. During FR20 components, the stimulus lights above both levers were illuminated and 20 lever presses produced a food pellet. During FI2 components, the houselight was illuminated and a single lever press following 2 min produced a food pellet. During timeout components, the chamber was dark and responses on the active lever produced a brief tone. A limited hold specified that if 3 FR20 components were not completed in 5 min or if a FI2 was not completed in 3 min, the schedule progressed to the next component. Sessions were conducted Monday–Friday at approximately the same time of day (1400 h).

Initially, rats were trained to lever press under an FR1 schedule of food presentation. When responding was maintained by food presentation, the response requirement was raised to FR20. Subsequently, the FI2 and timeout components were added. After approximately 80 sessions, responding under the FR20,FI2 schedule appeared stable. In this respect, stable responding was defined as less than 20% day-to-day variability in FR20 and FI2 response rates for at least five consecutive sessions. Additionally, daily cumulative response records were visually inspected to assure consistent responding. Rats were then divided into four treatment groups: 15 min ischemia + PD117302, 15 min ischemia + vehicle, no ischemia + PD117302, and, no ischemia + vehicle. Groups were balanced with respect to FR20 and FI2 response rates.

Production of Ischemia

Cerebral ischemia was produced using a four-vessel occlusion procedure as described by Pulsinelli and Brierly (31). Under anesthesia produced by inhalation of halothane (2.5%), in a combination of nitrous oxide (30%) and oxygen (70%), both vertebral arteries were cauterized at the level of the first cervical vertebra. Silastic tubing snares were placed around the left and right carotid arteries and exteriorized through the nape of the neck. After the wounds were treated and closed, rats were returned to their home cages for a 24 h recovery period.

On the following day, reversible carotid artery occlusion was produced by tightening the snares for 15 min. Occlusions were operationally verified by observation of immobility and by isoelectric bipolar cortical EEG recordings obtained from scalp needle electrodes placed over the frontal-parietal regions of the skull. Body temperature was maintained at 37°C during the occlusion period by a heating pad. Rats in the no ischemia groups were treated identically as rats in the ischemia groups except that the arterial snares were not tightened. Daily experimental sessions were continued following a 24 h recover period.

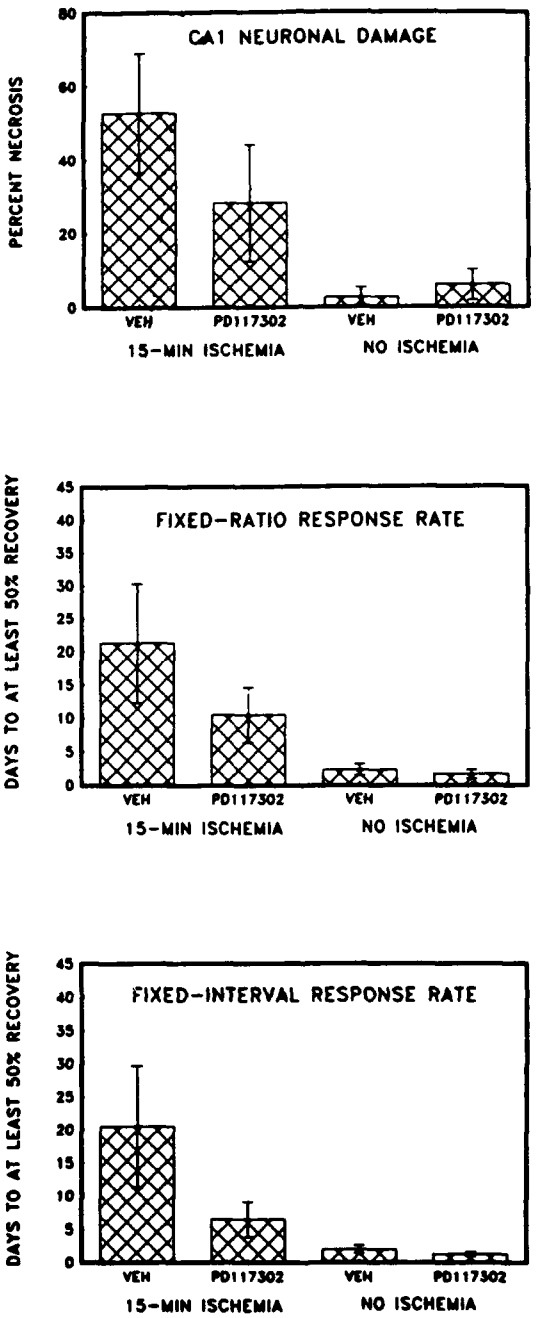

FIG. 1. Effects of 15 min 4-VO (left) or sham occlusion (right) on CA1 neuronal damage (top), and days to at least 50% recovery of responding under the FR20 (middle) and FI2 (bottom) components of the multiple schedule of reinforcement. Rats received PD117302 (n = 4) or vehicle (n = 4) following ischemia or PD117302 (n = 3) or vehicle (n = 3) following sham ischemia. Vertical lines inside the bars represent ± SEM.

TABLE 1

BASELINE RESPONDING UNDER THE SCHEDULE OF REINFORCEMENT

Group	N	FR20	FI2	FIQL	Session Responses
Ischemia + PD117302	4	213 ± 15	85 ± 8	50 ± 4	5796 ± 318
Ischemia + vehicle	4	175 ± 12	92 ± 8	53 ± 3	5817 ± 443
No ischemia + PD117302	3	154 ± 13	85 ± 4	58 ± 2	5166 ± 290
No ischemia + vehicle	3	212 ± 19	84 ± 9	60 ± 4	5652 ± 336

Values represent the mean ± SEM from five sessions preceeding treatments. Response rate values (FR20, FI2) are as responses per minute. Fixed-interval quarter-life (FIQL) values are as a percentage of the interval.

Histopathology

Rats were sacrificed 47 days after 4-VO by IP injections of a mixture of Ketamine (70 mg/kg) and Rompun (6 mg/kg) and the brains perfusion fixed with formaldehyde (10%) and processed for frozen section histology as previously described (12). Coronal sections (60 μm) were taken at 360 μm intervals at the level of the habenula and stained with cresylechtviolett (Nissl stain).

The area of neuronal degeneration within the dorsal hippocampus was quantitatively assessed at 63× using a Loats Image Analysis (LIA) system (Loats Associates, Westminster, MD). For each rat, the area of staining of the CA1 pyramidal cell layer was determined using a custom procedure developed for the LIA which utilized a densometric measurement of stained neuronal areas. For each section, the density of the defined area of CA1 staining was compared to the density of staining in the corresponding dentate gyrus (which is not significantly damaged) and corrected for background staining density. Results are expressed as a percentage of damage.

Pharmacological Procedure

PD117302 ((±)-trans-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzo[b]thiophene-4-acetamide) (Parke–Davis) was dissolved in saline (0.9%) and saline was used for vehicle injections. Injections were administered IP in a volume of 1.0 ml/kg body weight. Either 1.0 mg/kg PD117302 or vehicle was administered upon reperfusion (or the sham equivalence for the no ischemia treatment) and at 2, 4, and 6 h postocclusion. The dose of PD117302 was chosen on the basis of previous research in our laboratories (24).

Data Analysis

When a response under the schedule of reinforcement occurred, the elapsed time within the session was recorded. From these data the total number of responses, and the rate of responding (responses per minute), during each component of the schedule was calculated for each rat. To minimize the differences in response rates between subjects, these data were converted to a percentage of the average values obtained during the five sessions before treatment (baseline). From the normalized values, the number of days for responding to recover at least 50% of baseline values was calculated. Quarter-life measures were calculated to assess response distribution during the fixed-interval component. The quarter-life is defined as the proportion of the interval elapsed to emit 25% of the total responses in the interval. Thus, a quarter-life value of 0.25 suggests linear responding throughout the interval. To assess the difference between groups, a 2 × 2 ANOVA [(ischemia/no ischemia) × (PD117302/vehicle)] with multiple contrasts (t) was calculated using the General Linear Models procedure of the SAS (Cary, NC) statistical software package. Product–moment correlation coefficients were calculated to assess the relationships between behavioral and histological measures.

RESULTS

Three rats expired shortly after the induction of ischemia and two rats failed to meet the criteria for ischemia. None of these rats received injections of PD117302. Data from these rats were eliminated from analysis.

Transient ischemia produced significant necrosis in CA1 hippocampal cells (Fig. 1, top) as compared with rats in the no ischemia treatment, $F(1, 10) = 7.12$, $p < 0.05$. PD117302 decreased CA1 neuronal cell loss and the average necrosis in PD117302-treated rats was 28% as compared to 53% for vehicle-treated rats. Group comparisons indicated that histopathology scores for rats in the PD-117302 + ischemia treatments were not significantly different than those from rats in any other treatment group. In contrast, scores for rats in the vehicle + ischemia treatment were significantly different than both of the no-ischemia treatments ($p < 0.05$). No or minimal CA1 necrosis was observed in rats in either the PD117302 + no ischemia or vehicle + no ischemia treatment groups. The drug by treatment interaction for CA1 necrosis was not significant.

The multiple schedule of reinforcement produced distinctive rates of responding under the FR20 and FI2 components in all rats. Response rates under the fixed-ratio requirement were relatively fast and constant within the component. In contrast, response rates under the fixed-interval requirement were comparatively slower and positively accelerated within the component as evidenced by quarter-life values observed to be typically 50% or greater. Response rates during timeout components preceding the FI2 were very slow, while response rates during timeout periods preceding the FR20 were similar to the response rates observed during the FR20. Group means ± SEM for response rates during FR20 and FI2 components, and total session responses, from the five sessions preceding treatment, are presented in Table 1.

Transient ischemia produced substantial disruption of responding and, in general, responding was disrupted to the same extent in all components of the schedule of reinforcement. In most rats, performance degradation was characterized by a complete, or nearly complete, response suppression for a number of days, followed by a rapid return to near-baseline levels of responding. The pattern of responding in both the FR and FI components (i.e., response rate and quarter-life) also tended to return to near-baseline levels. Responding for one rat in the vehicle + ischemia treatment, however, remained completely suppressed over the duration of the 45-day posttreatment testing period. For purposes of analysis, this rat was assigned the maximum recovery

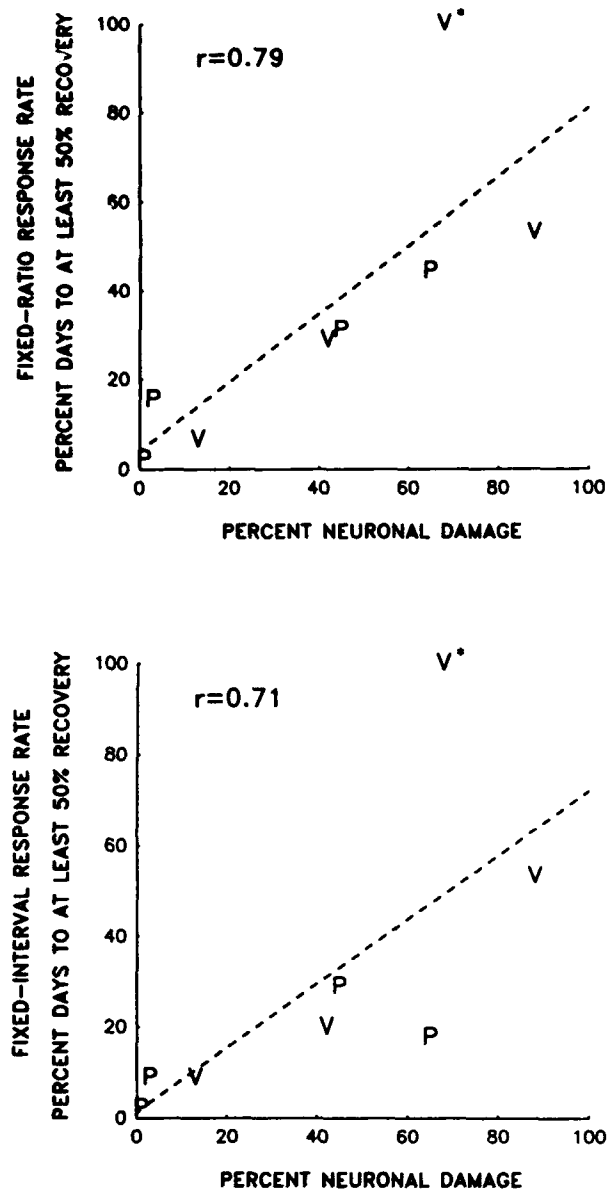

FIG. 2. Relationship between CA1 neuronal damage and time to recover at least 50% of baseline response rates (as a percentage of the 45 daily postischemic sessions) under the FR20 (top) and FI2 (bottom) components of the multiple schedule of reinforcement in rats following 15 min 4-VO. Symbols: P, rats treated with PD117302 following ishemia, V, rats treated with vehicle following ischemia. Values of r indicate product–moment correlation coefficients. Dashed diagonals represent regression lines. Asterisk indicates that response rate was less than 50% of baseline for the duration of the recovery period.

time score. During the postischemic period, body weights remained stable as all rats readily consumed postsession food (which occasionally included food pellets normally available only during experimental sessions). Some degree of response disruption was observed following surgical procedures (i.e., vertebral artery cauterization) for rats in the PD117302 + no ischemia or vehicle + no ischemia groups, but, responding recovered to near-baseline levels within a few days.

Comparisons between the number of posttreatment days before FR response rates recovered to at least 50% of pretreatment levels (Fig. 1, middle), indicated a significant effect for ischemia, $F(1, 10) = 5.57$, $p < 0.05$. PD117302 decreased FR recovery time following ischemia and the average number of days to recover to at least 50% of baseline levels in PD117302-treated rats was 10.5, as compared to 21.3 for vehicle-treated rats. Multiple contrasts indicated that FR recovery times for rats in the PD117302 + ischemia treatment were not significantly different than for any other treatment. In contrast, recovery times for rats in the vehicle + ischemia treatments were significantly different than for rats in both no ischemia treatments ($p < 0.05$). The drug-by-treatment interaction for FR recovery time was not significant.

Comparisons between the number of posttreatment days before FI response rates recovered to at least 50% of pretreatment levels (Fig. 1, bottom) indicated a nearly significant effect for ischemia, $F(1, 10) = 4.36$, $p < 0.06$). PD117302 decreased FI recovery time following ischemia and the average number of days to recover to at least 50% of baseline levels in PD117302-treated rats was 6.5 as compared to 20.5 for vehicle-treated rats. Multiple contrasts indicated that FI recovery times for rats in the PD117302 + ischemia treatment were not significantly different than for any other treatment. In contrast, recovery times for rats in the vehicle + ischemia treatments were significantly different than for rats in both no ischemia treatments ($p < 0.05$). The drug by treatment interaction for FI recovery time was not significant.

A substantial relationship between recovery from ischemia-induced disruption of performance under the multiple schedule of reinforcement and ischemia-induced CA1 necrosis was observed (Fig. 2). When recovery time was quantified as a percentage of the 45 day recovery period, significant positive correlations were observed for FR responding ($r = 0.79$, $p < 0.05$) and for FI responding ($r = 0.71$, $p < 0.05$).

DISCUSSION

In rats, 15 min forebrain ischemia, induced by 4-VO, produced significant CA1 hippocampal damage in a fashion consistent with previous studies. In general, the degree of necrosis was greater in the present study than we observed previously using a 5 min occlusion duration (12). Although the latter study used a different method to quantify histological damage, it is likely that the increased duration of ischemia produced greater damage. In this respect, it is notable that Buchan et al. (2) reported more severe and less variable CA1 damage using 15 min 4-VO as compared to a 5 min duration.

Although considerable variability between subjects was observed, in general, PD117302 was effective for reducing CA1 necrosis. This result is consistent with and extends results from a previous study (18) demonstrating neuroprotective efficacy of PD117302 in the gerbil. Statistically significant differences in CA1 necrosis were not observed between rats treated with PD117302 following ischemia and rats not subjected to ischemia. In contrast, significant differences were observed between rats treated with vehicle following ischemia and rats not subjected to ischemia. Thus, the degree of neuroprotection afforded by PD117302 in the present study, however, was limited because no statistical difference was observed between PD117302 and vehicle treatments for ischemia. Relatively few subjects were used in each group, and although the magnitude of neuroprotection necessary to observe a clear statistically significant difference was not obtained, the trend toward neuroprotection was observed. It is possible that the dose and frequency of administration of PD117302 used in the present study was less than optimal for neuroprotection. Further studies are needed to

establish the dose and time course relationships for PD117302 neuroprotection.

Ischemia produced substantial disruption of responding under the multiple schedule of reinforcement. Moreover, the degree of disruption was substantially greater than that observed previously using a 5 min occlusion duration (12). Unlike acute drug-induced disruption (e.g., 11), ischemia tended to completely suppress responding for a number of days. It is notable that during days when responding was suppressed, all rats would readily eat and no weight loss was observed. When responding recovered, it did so rapidly. Furthermore, the pattern of responding produced by the schedule recovered along with overall responding. In contrast, during training, a far greater number of sessions was required for the multiple schedule to control responding with respect to the distinctive rates and patterns observed in each component. Therefore, the ischemia produced a condition consistent with a temporary retrograde amnesia.

A significant correlation was observed between recovery time under either the FR20 or FI2 components of the schedule of reinforcement and CA1 necrosis following ischemia. Because hippocampal functions are generally believed to play a major role in memory and performance functions (8,22,28,37), this result suggests that schedule-controlled responding can characterize hippocampal injury, and thus, neuroprotective efficacy. Therefore, scheduled-controlled responding is a valuable complement to maze and avoidance procedures (6,39,41,42) already used to evaluate behavioral effects of ischemic injury.

Rats surgically prepared for 4-VO, but only sham occluded, also showed some degree of response disruption under the schedule of reinforcement. Recovery for these rats, however, was very rapid in every instance. Nevertheless, a certain degree of response disruption observed previously using 5 min occlusion (12) was probably due to surgical preparation, including vertebral artery cauterization.

PD117302 facilitated recovery from ischemia-induced response disruption under the schedule of reinforcement. That is, rats treated with PD117302 following ischemia tended to respond sooner than those treated with vehicle. Improvement in behavioral outcome following ischemia has been previously reported using another kappa opioid, U-50,488 (27). In general, facilitation of recovery occurred to the same extent under the FR20 and FI2 components of the schedule. There were no statistical differences in recovery time between rats treated with PD117302 following ischemia and those rats not receiving the ischemic episode. In contrast, those rats treated with vehicle following ischemia required significantly longer recovery times than rats not receiving ischemia. As with CA1 necrosis, however, differences in recovery times between vehicle and PD117302 postischemic treatments were not significant. This result also demonstrates that PD117302 facilitation of recovery was less than optimal.

The present study evaluated the treatment of ischemic injury, induced by 4-VO, in rats, using the selective kappa opioid agonist, PD117302. Ischemic injury resulted in CA1 hippocampal necrosis and was correlated with behavioral suppression under a multiple schedule of food reinforcement. Behavioral suppression was prolonged and nonspecific in that performance under both components of the schedule of reinforcement were affected. When behavioral performance resumed, the pattern of responding recovered rapidly. PD117302 tended to decrease CA1 necrosis and shorten behavioral recovery time. Further studies are planned to evaluate the time course and dose-effect functions for specific kappa opioid agonists to determine the conditions resulting in maximum therapeutic efficacy.

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In conducting the research described in this report, the investigators adhere to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense (para 4-3, AR 360-5).

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