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Hibernation-Induction Trigger. II. *In Vitro* Effects of Prairie Dog Plasma Albumin on Mouse Vas Deferens Contractility

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BRUCE, D. S., D. E. COX, S. K. CRANE, M. L. DENHOLM, R. J. DHYANCHAND, M. J. HAMPL, J. A. KARY, A. S. KROBER, P. R. OELTGEN, N. D. HORTON AND H. J. HARLOW. *Hibernation-induction trigger. II. In vitro effects of prarie dog plasma albumin on mouse vas deferens contractility*. PHARMACOL BIOCHEM BEHAV **58**(3) 627–630, 1997.—Involvement of opioid molecules in hibernation is well established, with the *delta* opioid receptor implicated in hibernation induction. Previous studies have shown that plasma albumin fractions (PAFs) from hibernating mammals contain an uncharacterized ligand called "hibernation-induction trigger" (HIT), which causes inhibition of induced contractility in the guinea pig ileum (GPI). In part I of this study, we described effects of PAF from two species of prairie dogs on induced contractility of the GPI. In the present study (part II), we examine the response of the mouse vas deferens (MVD), which is populated with the *delta* receptor subtype, to increasing concentrations of PAF from the white-tailed prairie dog (WT) and the black-tailed prairie dog (BT). Dose–response curves of lyophilized PAF yielded IC_{50} values (mg) (mean dose that inhibits contractility to 50% of control) of 11.0 for summer WT, 10.6 for hibernating WT, 9.4 for summer BT, 12.2 for winter active BT, and 4.7 for winter hibernating BT. These results suggest that *delta* opioid (HIT) is present in both species throughout the calendar year and that the induction of hibernation may involve not only levels of opioid but also dynamic interactions between endogenous opioid and its receptors. © 1997 Elsevier Science Inc.

Hibernation induction trigger HIT Prairie dog Mouse vas deferens

THE INDUCTION of hibernation in ground squirrels (*Spermophilus tridecemlineatus*) via infusion of blood, plasma or plasma albumin from hibernating ground squirrels indicates the presence of a circulating trigger that is responsible for the onset of hibernation (1–4). This hibernation-induction trigger (HIT) is present in plasma albumin fractions (5), is believed to exert its effects through opioid receptors (6,7), and its levels appear to change seasonally in mammalian hibernators (8–10). In part I of this study (12), we reported that both white-tailed and black-tailed prairie dogs' plasma albumin fractions (PAF) show significant opioid activity as assayed with the guinea pig ileum (GPI) longitudinal muscle/myenteric plexus model. We have demonstrated that [D-Pen^{2,5}]-enkephalin (DPDPE), a potent δ agonist, has a suppressing effect on GPI contractility, which is reversed by ICI-174,864, a selective δ antagonist (8), but, because the GPI possesses all three opioid receptor subtypes (μ, κ, δ), further elucidation of the action of prairie dog HIT demands a receptor-specific assay.

The mouse vas deferens (MVD) possesses δ opioid receptors well coupled to function, making it a more specific assay model (11) than the GPI. In the present study, the winter and summer levels of HIT in plasma albumin fractions (PAFs) were assayed in the same two prairie dog species studied in part I (12); these species are believed to have evolved from an ancestral lineage capable of spontaneous hibernation and may be related to ground squirrels (13). The white-tailed prairie

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dog (*Cynomys leucurus*) is an obligatory hibernator that enters torpor with circannual rhythmicity (14). However, the black-tailed prairie dog (*C. ludovicianus*) does not appear to enter torpor to the same extent and remains active throughout the winter unless it is without food and water (14). We address the question of whether selection pressures associated with the evolution of these two species since the Pliocene have resulted in a reduced biochemical capacity by the black-tailed prairie dog to hibernate. To test this hypothesis, PAFs obtained from both species in the summer and winter were examined for their effects on induced contractility of the MVD containing the unitary δ subtype of opioid receptor.

METHODS AND MATERIALS

Experimental Animals

Black-tailed and white-tailed prairie dogs, approximately 970–980 g body weight, were captured in the wild and maintained in captivity for the duration of the investigation. Blood samples were obtained via cardiac puncture. Active animals were first anesthetized with Ketamine HCl, 36 mg/kg IM; hibernating prairie dogs received no anesthesia. Blood was drawn from 9 white-tailed (WT 1–9) and 16 black-tailed prairie dogs (BT 1–16); prairie dogs BT 1–5 and 11–16 and WT 1–5 had blood drawn during the winter months, and prairie dogs BT 6–10 and WT 6–9 had blood drawn during the summer months. WT 1–5 were in natural hibernation when blood was drawn; BT 1–5 were active during the winter; and BT 11–16 were fasted and deprived of water in December or January after placement in a cold room, thereby inducing them to enter hibernation. Blood samples were obtained while BT 11–16 were in hibernation.

Albumin Fraction Preparation

Affinity chromatography with Affi-Gel Blue (Bio-Rad Industries) as the matrix was used to obtain a highly homogeneous PAF. A 15.5- × 1-cm affinity chromatography column was packed with 10 ml bead volume equilibrated with 0.02 M Na-phosphate buffer, pH 6.8. The albumin fraction was eluted with a 0.02 M Na-phospate and 1.4 M NaCl buffer, pH 5.7, after which it was lyophilized and stored at −75°C.

MVD Isolation and Assay

Male CFI mice (Sasco) weighing 30–40 grams were killed by cervical dislocation. Both vasa deferentia were removed and tied together (one in an anatomical position and one in an inverted position). The MVD were suspended in a 10-cc muscle chamber containing Mg^{2+}-free Krebs bicarbonate solution (NaCl 118 mM, $NaHCO_3$ 25 mM, glucose 11 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM) and continually aerated with 95% O_2–5% CO_2 at 37°C. Contractions of the MVD were induced by field stimulation via two platinum coil electrodes (stimulus parameters: 100 V, 1–2 ms pulse duration, (0.16 Hz) and recorded with a Grass FT03 transducer (Grass Instrument Co., West Warwick, RI) on a Gould 3400 thermal

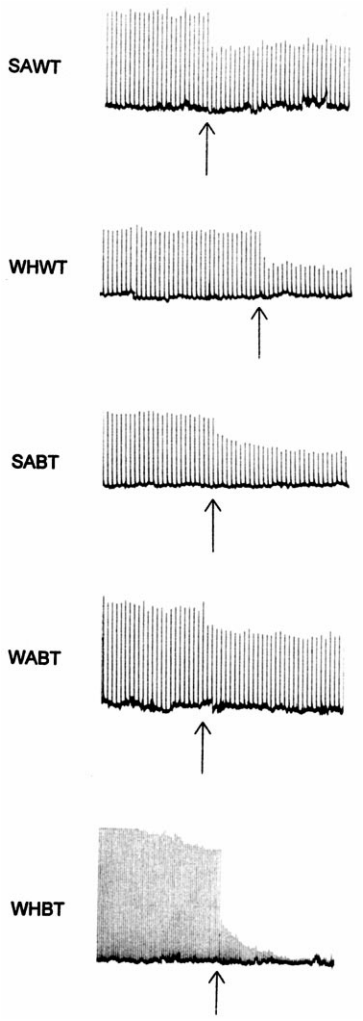

FIG. 1. MVD contraction recordings. (Arrows indicate points at which 15 mg PAF samples were added to the contracting MVD.)

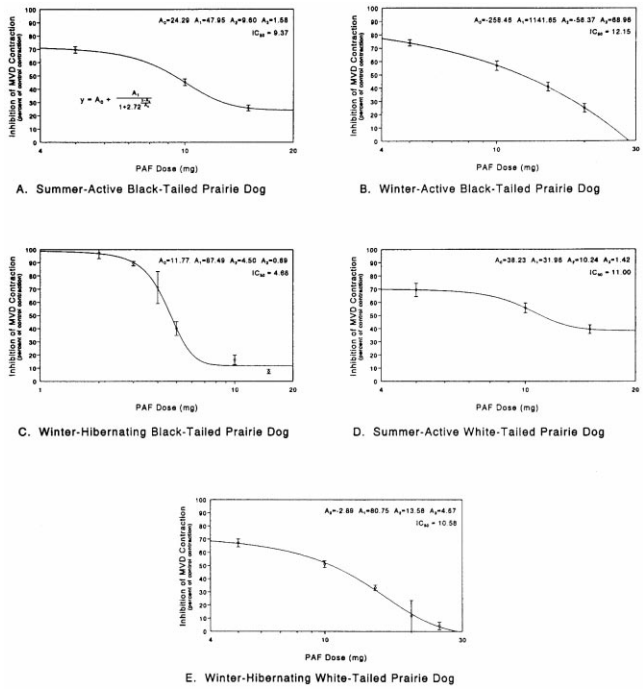

FIG. 2. Mean dose-response curves.

trace recorder (Gould Inc., Cleveland, OH). A resting tension of approximately 125 mg was maintained on the MVD at all times. After isolation, the MVD were allowed to equilibrate for 45 min, with Krebs bicarbonate rinses every 5 min.

A dose–response study was performed using 5-, 10- and 15-mg samples of summer-active black tail (SABT), winter-active black tail (WABT), winter-hibernating black tail (WHBT), summer-active white tail (SAWT), and winter-hibernating white tail (WHWT) PAFs. With some groups, sufficient PAF made it possible to do additional dose-response studies at 20 mg (WABT), at 2, 3, and 4 mg (WHBT), and at 20 and 25 mg (WHWT). PAFs from each group were assayed a minimum of 18 times for their ability to inhibit MVD contractility. After each trial, contractions were restored to control levels with three Krebs bicarbonate rinses at 5-min intervals. The average inhibition of contraction for each albumin dose was used to construct a curve. A dose–response curve was obtained with a nonlinear curve-fitting program, after which the equation was solved for the IC_{50} value (the level of albumin that inhibits the MVD contraction to 50% of control contraction height).

RESULTS

Figure 1 shows a representative series of MVD contraction recordings as used in the construction of dose–response curves for all prairie dogs used in the study. Arrows indicate the addition of 15-mg PAF samples to the muscle chamber. Figure 2 shows the dose–response curves for each of the five different prairie dog albumin sample groups. The average IC_{50} values (mg, with SEM) were 11.0 for SAWT, 10.6 for WHWT, 9.4 for SABT, 12.2 for WABT, and 4.7 for WHBT. Figure 3 is a comparison of the inhibition of contractility at 5, 10, and 15 mg PAF for SAWT, WHWT, SABT, WABT and WHBT (mean ± SEM). Figure 4 is a summary of the IC_{50} values (mg) for the five different prairie dog plasma albumin sample groups. Table 1 displays results of unpaired *t*-tests between prairie dog groups for MVD contractility suppressions by 5-, 10- and 15-mg samples of PAFs. Many differences are significant.

DISCUSSION

Our studies suggest that the HIT molecule is present in both summer and winter white- and black-tailed prairie dogs.

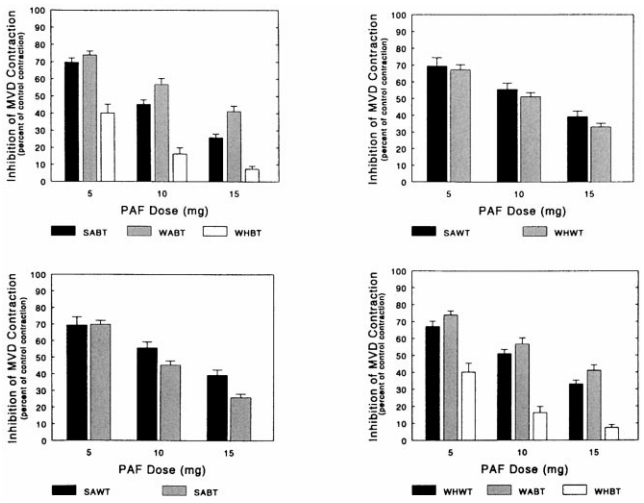

FIG. 3. Comparison of MVD contractility suppression by 5, 10, and 15 mg samples of prairie dog plasma albumin fractions.

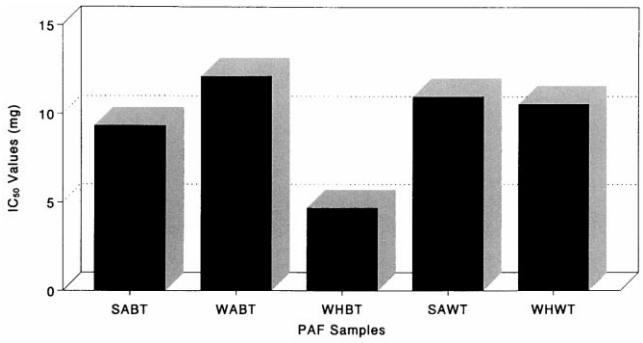

FIG. 4. IC_{50} values for prairie dog plasma albumin fractions.

As expected, there was a greater amount of depression in the contractility of the vas deferens with WHWT albumin fraction than with the SAWT fraction (see Figs. 3 and 4 and Table 1), although differences were not significant. The same was true when comparing contractility suppression between WHBT and WABT fractions, but here the differences are significant ($p \leqslant 0.01$). The WHBT fraction is from black-tailed prairie dogs that do not hibernate naturally, although they possess the physiological ability to do so when without food and water during the winter. For this study, they were *induced* to hibernate by placement in a cold room and withholding food and water. Their plasma albumin was the most potent in inhibiting vas deferens contractility, supporting the hypothesis that ani-

TABLE 1

RESULTS OF UNPAIRED *t*-TESTS COMPARING MVD CONTRACTILITY AT 5-, 10- AND 15-MG DOSES OF PRAIRIE DOG PAFs

PAFs (mg) Compared	Significant/ Not Significant	$p \leqslant$
SABT vs. WHBT		
5 mg	S	0.01
10 mg	S	0.01
15 mg	S	0.01
WABT vs. WHBT		
5 mg	S	0.01
10 mg	S	0.01
15 mg	S	0.01
SAWT vs. WHWT		
5 mg	NS	—
10 mg	NS	—
15 mg	NS	—
SABT vs. SAWT		
5 mg	NS	—
10 mg	S	0.05
15 mg	S	0.01
WHBT vs. WHWT		
5 mg	S	0.01
10 mg	S	0.01
15 mg	S	0.01
SABT vs. WHWT		
5 mg	NS	—
10 mg	NS	—
15 mg	S	0.05

mals in hibernation possess a greater concentration of HIT containing δ opioid. However, in comparing SABT with WHWT, the mean MVD contractilities were not significantly different at 5- and 10-mg dosages but were significantly different at 15 mg ($p \leqslant 0.05$). This leads us to believe that the concentration of HIT molecules in both SABT and WHWT are similar. In nature, SABT represents blood from animals in an active state, not in hibernation, whereas WHWT is from animals in hibernation. As discussed in the previous report (12), comparisons between species are not sound physiologically because HIT is not yet characterized biochemically, and albumins from different species are most certainly different in composition. Thus, there could be a change in the concentration of receptors or a conformational change of the receptor site itself, allowing for the binding of HIT molecules rather than just a change in HIT concentration. Although this cannot be ruled out, firm conclusions cannot be drawn until purified HIT compound can be studied. In addition, there may be a competing molecule to HIT that is seasonally present (3), perhaps also bound to the albumin fraction.

When examined on an *intraspecies* basis, it does appear that there is a correlation between physiological state and the capacity of prairie dog plasma albumin to suppress MVD contractility. In the white-tailed prairie dog, WHWT fraction is a more potent suppressor than SAWT fraction (although the differences are not significant), and WHBT fraction inhibits much more effectively than either SABT or WABT fractions (Fig. 4, Table 1). On the basis of the bioassays performed in this study, PAF (HIT) from the hibernating black-tailed prairie dog has the highest concentration of δ opioid of all animals studied. Therefore, these data do not suggest that the black-tailed prairie dog has evolved a reduced PAF (HIT) and opioid activity but can hibernate in nature when exposed to the proper stimuli. Indeed, in previous studies, no significant differences have been described between these two species in terms of kidney structure and function (15), brown adipose tissue and seasonal fat content (16) or fatty acid saturation (17), all believed to be essential for effective hibernation. However, the black-tailed prairie dog does not seem to become spontaneously anorexic or to conserve protein during induced fasting like the white-tailed prairie dog (16), suggesting that some distinct biochemical differences do exist between these two species which may influence their different strategies for winter survival.

Current work by two of us (P. R. O. and N. D. H.) involves separating HIT peptide from the albumin and determining its amino acid sequence. Identifying the structure of HIT will facilitate further investigation of its site of production, mechanism of action and means of regulation.

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