AD-A171 960 COMPARISON OF PFDA (PERFLUORO-N-DECANOIC ACID) AND TCDD ON HEART MEMBRANES(U) WRIGHT STATE UNIV DAYTON OH SCHOOL OF MEDICINE A E LANGLEY 18 JUN 86 1/1

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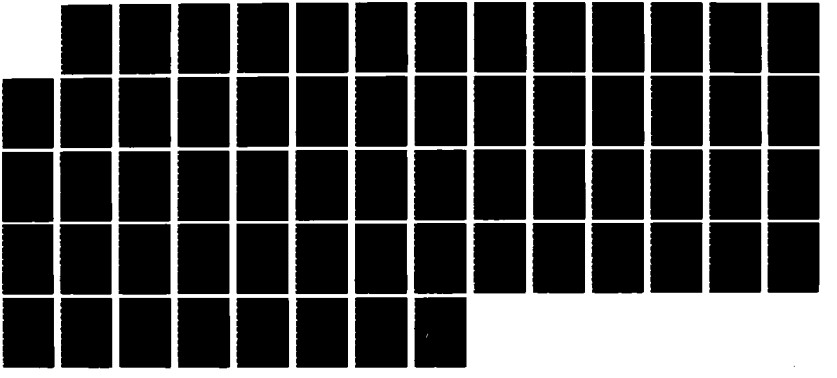

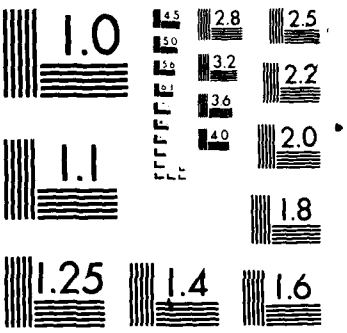

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Our initial observations of a reduced responsiveness to adrenergic stimulation of hearts from PFDA-treated rats has been examined in terms of functional components of the myocardial membrane. The reduced responsiveness can be explained in part by a decrease in the number of cell surface beta adrenergic receptors. An early and dramatic fall in serum levels of thyroid hormones was observed. Because much of the loss of adrenergic responsiveness can be explained by the effects of PFDA on thyroid hormones we concentrated our research on the antithyroid actions of PFDA. The most significant results of this research was the observation (reported in detail herein) that thyroxine supplementation could prevent the hypophasia characteristic of PFDA treatment without effecting the body wasting syndrome and hypothermia. The mechanism of these paradoxical actions remains to be determined.

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FINAL TECHNICAL REPORT

AFOSR-TR- 86-0651

THYROID AND METABOLIC EFFECTS OF PFDA IN RATS

We have observed dramatic effects of PFDA on a number of cardiac parameters. A single 75 mg/kg ip injection of PFDA produced a decrease in resting heart rate and reduced the inotropic response to nerve stimulation or norepinephrine infusion in isolated rat hearts (see enclosed manuscript #1). The number of beta adrenergic receptor binding sites in rat heart membranes was reduced by PFDA treatment (see enclosed manuscript #1) and the ability of norepinephrine to stimulate cardiac membrane adenylate cyclase was also reduced by PFDA treatment (see enclosed manuscript #2). These alterations in cardiac membrane functions were accompanied by significant falls in body temperature beginning 4 days after PFDA treatment (see enclosed reprint).

Since the cardiac and hypothermic effects of PFDA projected a picture of clinically hypothyroid animals we measured circulating levels of thyroxine (T_4) and triiodothyronine (T_3). Both T_4 and T_3 were significantly decreased by PFDA treatment (see enclosed reprint). These effects persisted for as long as 60 days after a single injection of PFDA.

A dose - response curve of the serum thyroid hormone lowering effects of PFDA was carried out. Table 1 shows a dose dependent fall in both serum T_4 and T_3 24 hours after exposure to an ip. injection of PFDA.

In an effort to determine the mechanism(s) of this thyroid hormone lowering action of PFDA we carried out the following experiments:

1. Response of the thyroid gland to TSH stimulation. Animals were dosed with PFDA or propylene glycol (PG) at 10:00 a.m. and challenged 12 hours later (10:00 p.m.) with TSH. This time sequence was chosen because rat serum TSH reaches the lowest point in its diurnal cycle at 10:00 p.m.[1] and will therefore, contribute little to thyroid stimulation by exogenous TSH. Figure 1 shows that control rats had a 46% increase in serum T_4 levels at eight hours while PFDA treated rats showed a 50% increase (NS). The greatest increase in total T_4 occurred 12 hours after TSH (24 hours after PFDA). Sixteen hours (28 hours after PFDA) after TSH serum T_4 had fallen to levels similar to those seen eight hours after TSH (20 hours after PFDA treatment).

   Eight hours after TSH serum T_3 levels were increased by 115% in untreated controls, and by 80% in PG controls (NS). In PFDA rats serum T_3 increased 240% which was significantly greater than either control group. Twelve hours after TSH (24 hours after PFDA) serum T_3 levels were the same as those seen in the controls at 8 hours. By 16 hours after TSH the T_3 was still 50% greater than pre-TSH levels. These data demonstrate that the thyroid glands in PFDA-treated rats are capable of responding to a TSH challenge in a relatively normal fashion.

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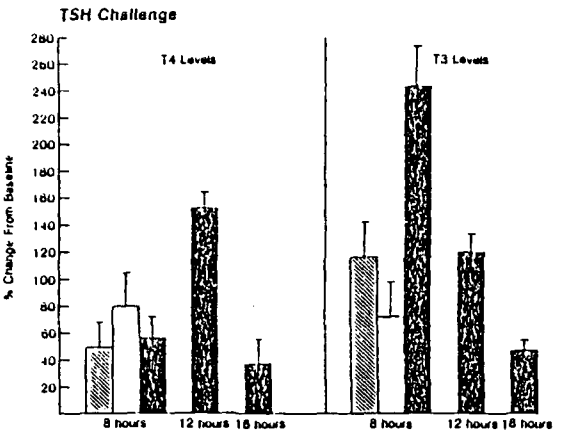

Figure 1. Percent change from baseline in serum T_4 and T_3 in rats challenged with TSH. PFDA □, n=5; PG control □, n=5; untreated controls □, n = 5.

2. Response of the thyroid gland to TRH stimulation. Rats were dosed with PFDA or PG and challenged 15 or 22 hours later with 500 micrograms/kg synthetic TRH. Six animals were sacrificed two and six animals were sacrificed nine hours after TRH administration (24 hours after PFDA) and their sera assayed for total T_4 and T_3. Figure 2 show that there were no significant differences at either time point between PFDA-treated and propylene glycol control rats. These data demonstrate that the pituitary gland is also capable of responding normally to a TRH challenge.

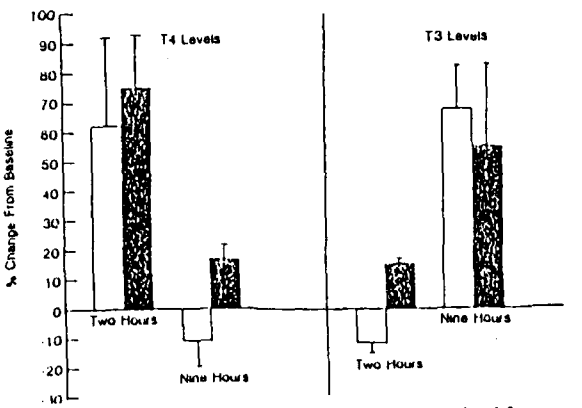

Figure 2. TRH challenge. Bars represent the percent change from baseline of serum T_4 and T_3 in PFDA-treated rats, □, n=6 or propylene-glycol control rats, □, n=3.

3. Radioiodine (I^{125}) uptake by the thyroid gland. In order to test the ability of the thyroid gland to trap iodine, rats were injected with 5 microcuries of carrier-free Na ^{125}I ten hours after a single injection of PFDA (75 mg/kg) or propylene glycol. Thyroid glands and sera were collected two hours after the Na ^{125}I administration and the gland to serum ratios of radioactivity were determined. The ratio in control rats was 75.3 $\pm$ 9.2 compared to 108.5 $\pm$ 13.7 in PFDA treated rats (NS). These data indicate that glands from PFDA-treated rats were capable of concentrating radioiodine to the same extent as thyroid glands from PG controls. It would appear from these data that the decrease in total serum T_3 and T_4 is not due to a loss of functional integrity of the thyroid or the pituitary gland.

4. Thyroxine supplementation. A group of 10 rats was treated daily with 200 mg/kg T_4 for 10 days prior to PFDA. Daily treatment with T_4 continued for 14 days after PFDA dosing. A group of 10 rats received 200 mg/kg T_4 alone and a group of 10 rats received PFDA alone. Figure 3 shows that rats treated with PFDA alone had an 80% decrease in food consumption by 3 days following PFDA. The average daily food consumption started to increase by day 6. These animals lost a maximum of 27% of their body weight by the 10th day after PFDA. These values are similar to those previously reported.[2] Rats supplemented with T_4 and treated with PFDA showed only a 21% maximum decrease in food consumption by the 4th day following PFDA. The average daily food intake began to increase on day 5. In some rats daily food consumption actually increased to greater than twice controls (55-60 gm/day). The average maximum total body weight loss occurred at day 13 and amounted to 24%. The results of these experiments demonstrate that it is possible to separate the effect of PFDA on food consumption from the effect on body weight loss.

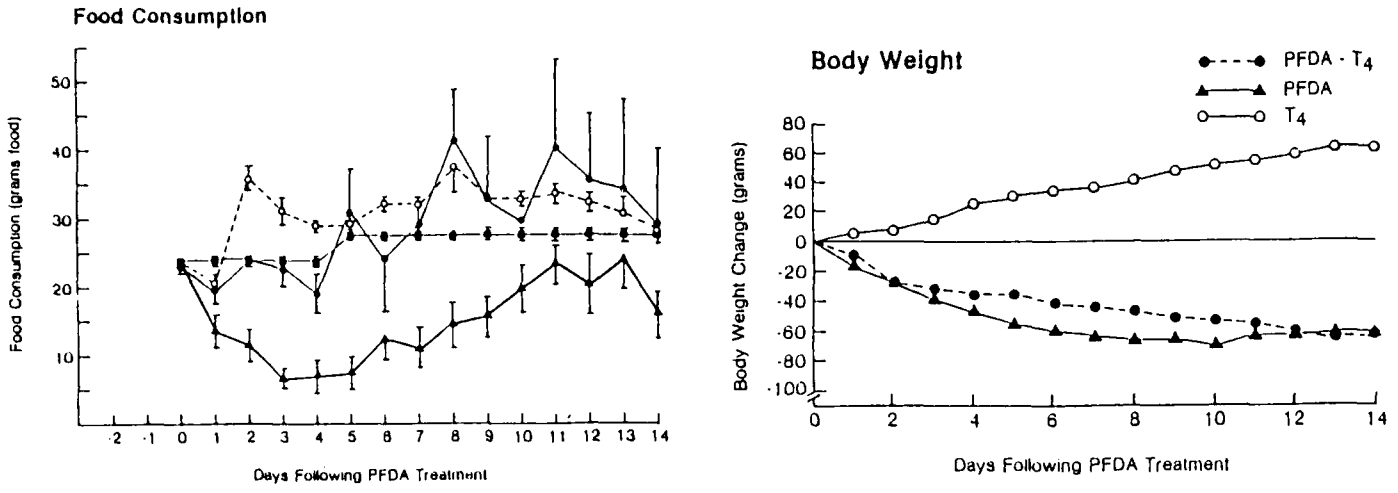

Figure 3. Food consumption and body weights in control rats, ■, n = 6; PFDA-treated rats, ▲, n = 8; T_4-treated rats, O, n = 6 and PFDA + T_4-treated rats, ●, n = 8.

Rectal body temperatures were also measured in these same animals. Thyroxine treatment alone produced a characteristic hyperthermic response (Figure 4). PFDA produced a hypothermia similar in magnitude to that previously reported (see enclosed reprint). Thyroxine supplementation delayed somewhat the hypothermic response to PFDA but did not significantly alter its magnitude.

These data indicate that in spite of normal to greater than normal food consumption, PFDA in the presence of thyroxine supplementation still produces a characteristic wasting syndrome and hypothermia.

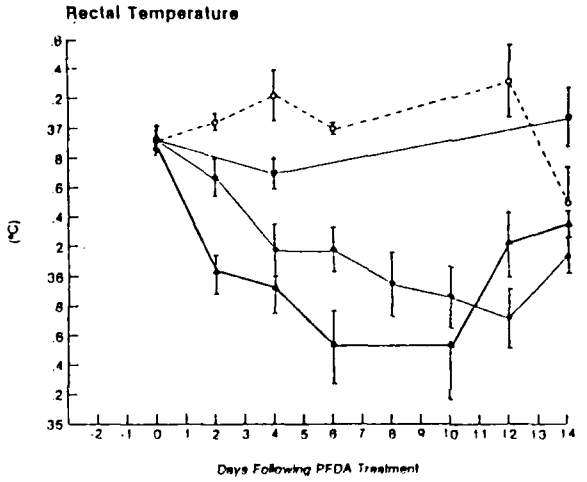

Figure 4. Rectal body temperatures in control rats, ■, n = 6, PFDA-treated rats, ▲, n = 8, T_4-treated rats, O, n = 6, and PFDA-T_4-treated rats, ●, n = 8.

Our observations that decreased food consumption and body weight loss with hypothermia produced by PFDA treatment can be differentially effected by thyroxine (T_4) administration suggests that these phenomena may involve separable perhaps independent actions of PFDA. It appears likely that PFDA rapidly alters some process(es) in the body that leads to decreased circulating levels of thyroid hormones apparently resulting in or contributing to anorexia. The wasting syndrome observed with PFDA treatment is therefore only partly due to the anorexia. A significant portion of the body weight loss may be due to separate biochemical (most likely metabolic) action(s) of PFDA.

Our current research 1) continues our efforts to determine the site(s) of the thyroid effects of PFDA; 2) explores potential mechanism(s) responsible for the wasting syndrome associated with PFDA treatment, and 3) attempts to define the role of the thyroid hormones in the anoretic response to PFDA.

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THYROID, BRADYCARDIC AND HYPOTHERMIC EFFECTS OF PERFLUORO-n-DECANOIC ACID IN RATS

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A single ip injection of perfluoro-n-decanoic acid (PFDA) to male Wistar rats resulted in an initially rapid, then gradual decrease in food consumption and a parallel loss of body weight. Body temperatures and resting heart rates were significantly depressed by PFDA treatment. As early as 12 h following a single dose of PFDA, serum thyroxine (T_4) levels were significantly reduced and remained depressed throughout the 8 day study. Serum triiodothyronine (T_3) was reduced by 35% 12 h following PFDA treatment and remained at that level throughout the study. These preliminary data suggest that an action on the thyroid axis may be an early primary response to PFDA and that some of the observed subsequent effects may in part be secondary to the change in thyroid hormone levels.

INTRODUCTION

Perfluoro-*n*-decanoic acid (PFDA) is a member of a family of perfluorocarboxylic acids that are used in a variety of industrial applications. These include film-forming foams for fire extinguishants, wetting agents, corrosion inhibitors, and for electroplating (Olson, 1983). They have also been used to impart water and oil resistance to leather, certain fabrics, and food wrapping paper (Rozner, 1980).

There have been reports of human exposure to perfluorinated carboxylic acids primarily in workers in fluorochemical plants (Ubel et al., 1980). The study demonstrated that these compounds persisted in the body long after exposure had been discontinued. While no apparent toxicities were reported with these acute exposures, the persistent nature of this class of chemicals may produce long-term effects.

Toxicity has been reported in laboratory animals, especially the rat. The gross toxicity produced by PFDA includes hypophagia, dramatic weight loss, and delayed lethality. The LD50 in male Fisher-344 rats was 64 mg/kg (Olsen 1983). The LD50 in male Wistar rats is around 75 mg/kg (M. E. Andersen, personal communication). The acute gross toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is also characterized by hypophagia, dramatic weight loss, and a delayed lethality (Seefeld and

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Peterson, 1982). Recently, Potter et al. (1983) showed that TCDD treatment produced a significant reduction in body temperature and a decrease in serum thyroxine (T_4) levels. Because of the similarity in the gross toxicity of PFDA to that of TCDD (Andersen, 1981), it was of interest to investigate the effect of PFDA on body temperature and serum thyroid hormone levels. In addition, the effect of PFDA on resting heart rate was also measured.

METHODS

Groups of male Wistar rats (200-250 g) were obtained from Harlan Industries and maintained in a constant-temperature environment (23.3°C, range 22.1-24.4°C) for 6 d prior to use. PFDA-treated animals received a single 75-mg/kg ip injection of PFDA in propylene glycol. One group of 30 rats was treated with PFDA at 5:00 p.m. on d 0, and daily food consumption and body weights were recorded over a period of 8 d. At 1 d after the PFDA-treated rats were dosed, a group of 30 weight-matched control rats was injected with propylene glycol at 5:00 p.m., pair-fed to the PFDA-treated group, and body weights were recorded. A group of 8 rats fed ad libitum were injected with propylene glycol at 5:00 p.m. on d 0 and their food consumption and body weights recorded for 8 d. All animals were weighed between 4:00 and 5:00 p.m. daily and then offered food. At different intervals beginning 12 h after dosing (5:00 a.m.), five rats (PFDA-treated or pair-fed) were sacrificed by decapitation and blood was collected in centrifuge tubes on ice, allowed to coagulate, and centrifuged at 3000×g for 10 min. The serum was stored at −20°C for triiodothyronine (T_3) and T_4 determinations. Ad libitum control rats were also sacrificed on d 0, 1, and 2 for determination of serum T_3 and T_4.

Rectal body temperatures were recorded at 2:00 p.m. daily for 8 d in a group of 17 PFDA-treated, 17 pair-fed control rats, and 8 ad libitum controls. A rectal thermometer was inserted to a depth of 3 cm, and the temperature was recorded on a Yellow Springs Instrument Company telethermometer.

Separate groups of eight PFDA-treated and eight pair-fed control rats were used to determine heart rates. Between 4:00 and 5:00 p.m. on alternate days beginning 2 d after dosing, the rats were lightly anesthetized with ether and needle electrodes were inserted under the skin of both front limbs and the right hind limb. The electrodes were connected to an ECG/Biotach Amplifier on a Gould 2400 Physiological recorder, and lead I of the electrocardiogram and instantaneous heart rate were recorded. Stable periods of heart rate recorded as the animals were beginning to emerge from the anesthesia were reported.

Total T_4 and total T_3 were determined by radioimmunoassay using assay tubes coated with antibody to T_4 or T_3 (Diagnostic Products Corpo-

ration, Los Angeles, Calif.). Concentrations of PFDA up to 100 μg/ml did not interfere with the radioimmunoassays for either T_4 or T_3.

All animals were handled in identical fashion and were fasted for 24 h prior to sacrifice. When sacrifice of PFDA-treated and control rats occurred on the same day, the sacrifice routine was randomized.

An analysis of variance (ANOVA) was carried out on the data to determine statistical differences among the groups. A Duncan's multiple range test was used to determine significant differences at the 0.05 level between pair-fed controls and PFDA-treated rats.

RESULTS

Food consumption decreased rapidly in the first 24 h and tapered gradually to 0 g/d by d 8 (Fig. 1). Body weights of the PFDA-treated rats fell from a pretreatment average of 250 g to 160 g at 8 days following PFDA treatment (Fig. 2). The pair-fed group of rats showed a similar weight loss with final weights around 190 g (Fig. 2).

One day following treatment the body temperatures of PFDA-dosed rats were significantly higher than pair-fed controls. Beginning on d 3 and continuing through the end of the study, the body temperatures of PFDA-treated rats were significantly lower than the body temperatures of pair-fed control rats (Fig. 3).

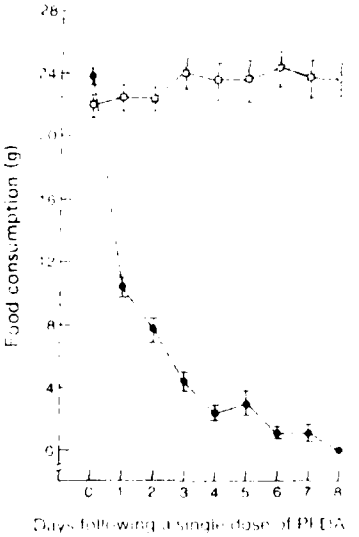

FIGURE 1. The effect of PFDA (75 mg/kg) on the amount of standard laboratory food (g) consumed by rats during a 24 h period. □, control, $n = 8$; ●, PFDA, each point representing the mean ± standard error of the mean (SEM) of a variable number of animals as follows: d 0 = 30, 1 = 25, 2 and 3 = 20, 4 and 5 = 15, 6 and 7 = 10, and 8 = 5.

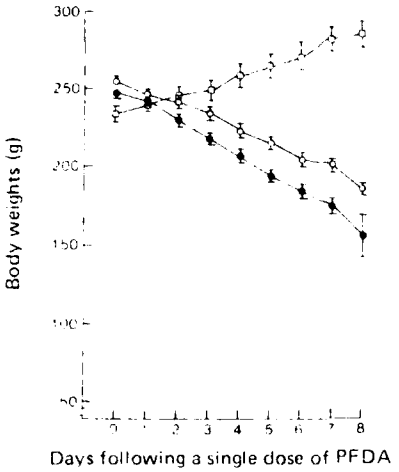

FIGURE 2. The effect of PFDA (●) and food restriction (pair-feeding, ○) on body weights of rats. Each point represents the mean ± SEM of a variable number of animals. Refer to legend of Fig. 1 for details. Ad libitum controls (□), $n = 8$.

Food restriction in the pair-fed controls did not alter resting heart rates. PFDA-treated rats showed a progressively lower resting heart rate with time after dosing. By d 6 the recorded heart rates were significantly lower in the PFDA-treated rats than in pair-fed controls (Fig. 4).

As early as 12 h following dosing with PFDA, serum thyroxine (T_4) levels were significantly reduced compared to pair-fed controls (Fig. 5). Serum T_4 levels continued to fall reaching a minimum value by d 2 and continuing at that level through d 8. Serum T_4 levels were significantly lower than pair-fed contro's at all tested times.

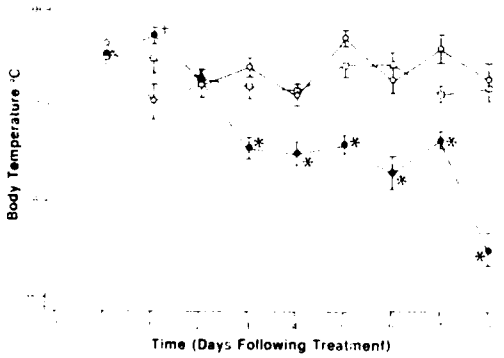

FIGURE 3. The effect of PFDA (●, $n = 17$) and pair-feeding (○, $n = 17$) on rectal body temperatures of rats. *, significantly lower than pair-fed controls, $p = 0.05$; †, significantly higher than pair fed controls, $p = 0.05$; □, ad libitum controls, $n = 8$.

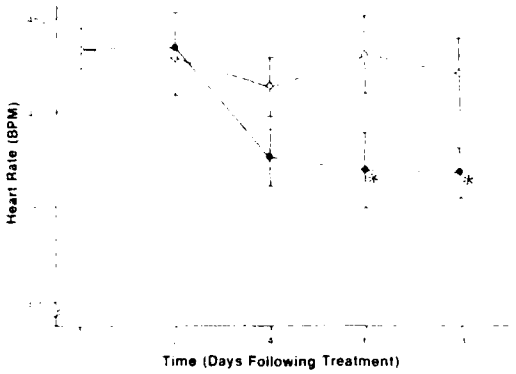

FIGURE 4. The effect of PFDA (●, $n = 8$) and pair-feeding (○, $n = 8$) on resting heart rates in rats. Controls (▽, $n = 16$). *Significantly less than pair-fed control, $p < .05$.

Serum triiodothyronine (T_3) showed a similar but less dramatic response to PFDA treatment (Fig. 6). By 12 h, circulating levels of T_3 were reduced by about 35% in PFDA-treated rats compared to pair-fed controls (72 versus 110 ng/dl). Serum T_3 values remained reasonably constant until d 8, when they fell to 50 ng/dl. Serum T_3 values were significantly lower than pair-fed controls only at 12 h, 1 d, and 2 d.

DISCUSSION

A single ip injection of PFDA (75 mg/kg) produced hypophagia and dramatic weight loss similar in magnitude but with a shorter time course than that previously reported with PFDA at 50 mg/kg (Olson and Andersen, 1983). Food restriction by pair-feeding a group of control rats produced a similar weight loss.

Significant decreases in body temperatures and heart rates occurred in the PFDA-treated rats when compared with pair-fed controls. The data suggest that PFDA may be affecting basic endogenous mechanisms

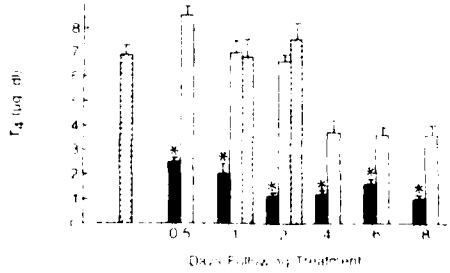

FIGURE 5. Effect of PFDA (solid columns) and pair-feeding (open columns) on rat serum thyroxine (T_3). Each bar is the mean ± SEM of five serum samples. Ad libitum controls (hatched column, d 0, $n = 15$; d 1, $n = 5$; d 2, $n = 5$). *Significantly less than pair-fed controls, $p < 0.05$.

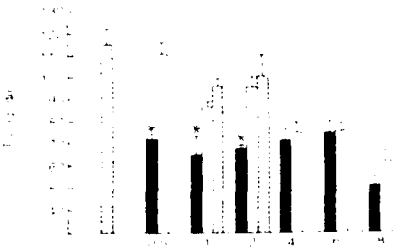

FIGURE 6. Effect of PFDA (solid columns) and pair-feeding (open columns) on rat serum triiodothyronine (T_3). Refer to legend of Fig. 5 for details.

that regulate these physiological functions. Hypothyroid individuals have cold extremities and are cold-sensitive (Ibbertson, 1979), probably due to metabolic alterations that decrease the rate of heat production (Mazzaferri, 1980). The thyroid hormones are reported to influence thermogenesis through an action on ATPase-mediated active sodium transport (Edelman and Ismail-Beigi, 1974; Himms-Hagen, 1976). In addition, thyroid status can affect cardiovascular function. Impaired myocardial contractility (Buccino et al., 1967) and bradycardia (Mazzaferri, 1980) are common manifestations of hypothyroidism. Therefore, we felt that many of the symptoms of PFDA treatment might involve alterations of thyroid hormone levels. Figures 5 and 6 show that as early as 12 h after a single dose of PFDA serum levels of thyroxine and triiodothyronine are significantly lower than controls. This occurs at a time when the animals do not demonstrate any overt signs of PFDA toxicity. Following 4 d of pair-feeding, serum T_3 values were at the same level as that produced by PFDA treatment; however, serum T_4 levels in PFDA-treated rats were significantly lower than in those pair-fed throughout the study. These data indicate that the depression of thyroid hormone levels produced by PFDA is not solely a result of starvation. It is nearly impossible to control all of the numerous influences that can alter circulating levels of thyroid hormones. However, since both groups of animals were handled identically, the fact that serum levels of both thyroid hormones are dramatically decreased as early as 12 h following a single ip injection of PFDA suggests that this may be an important observation in the response to PFDA treatment. The subsequent changes in body temperature and heart rate may be at least partly secondary to the alterations of circulating thyroid hormones. It is not clear from this study whether PFDA is acting directly on the thyroid gland or on the pituitary gland to depress TSH or on the hypothalamus to depress TRH. Since these data are only preliminary, additional experiments are in progress to better define these actions and to determine the primary site(s) of action of PFDA in producing these effects.

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MANUSCRIPT
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The Effects of Perfluoro-n-decanoic Acid (PFDA) in the Rat Heart.[1]

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Short Title

PFDA in Rat Hearts

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ABSTRACT

The Effects of Perfluoro-n-decanoic Acid in the Rat Heart. Pilcher, Gary D. and Langley, Albert E. (1986) Toxicol. Appl. Pharmacol.

Perfluoro-n-decanoic acid (PFDA) is a synthetic chemical resembling a 10 carbon fatty acid. Several studies have suggested that the toxic mechanism of PFDA may involve impaired lipid metabolism and/or altered cell membrane function. We examined the possibility that altered cell membrane structure in the heart might lead to changes in the functional activity of the organ. Functional characteristics were determined in the isolated perfused rat heart by measuring the ability of the heart to respond to either sympathetic nerve stimulation or infused norepinephrine. PFDA reduced the intrinsic resting heart rate and the inotropic response to a stimulus with maximal effects occurring 8 days after dosing. In addition, resting heart rate measured in vivo was found to be reduced in PFDA treated rats 6 to 8 days after dosing.

Beta-receptor binding studies conducted 8 days after a single dose of PFDA showed that the maximum binding capacity was reduced by PFDA treatment without significant changes in receptor affinity. It is concluded that the reduction in the inotropic response to catecholamines following PFDA treatment may be explained in part by lower beta-receptor density in the myocardial cell membrane. These effects may be related to the early fall in serum thyroid hormone levels previously reported.

INTRODUCTION

Perfluoro-n-decanoic acid (PFDA; nonadecafluorodecanoic acid, $C_{10}F_{19}O_2H$) is a straight-chain 10 carbon carboxylic acid with fluorine substituted for all hydrogens at the C-2 through C-10 carbon atoms. Straight-chain perfluorocarboxylic acids 8 to 12 carbons in length and structurally similar perfluorinated derivatives have a broad range of commercial application. They are used in electroplating and to impart water and oil resistance to fabrics, leather, and food wrapping paper (Bryce, 1964). In addition, the surfactant nature of perfluorinated fatty acyl compounds allows them to be used in the aqueous polymerization of fluorinated monomers (Griffith and Long, 1980) and in aqueous film-forming foams used in fire extinguishant mixtures (Shinoda and Nomura, 1983).

Many perfluorinated compounds are chemically inert (Clarke et al. 1973) and some are reported to be persistently retained in experimental animals for significant time periods following exposure (Clarke et al. 1970). In addition, certain perfluorinated carboxylic acids have been found in the serum of fluorochemical workers long after exposure had been discontinued (Ubel et al. 1980). In this study, the authors noted that no adverse health effects were apparent among the workers though the persistent nature of these chemicals might result in long-term toxicity.

The acute toxicity of PFDA includes hypophagia with severe weight loss and delayed lethality. Olson and Andersen (1983) reported an LD 50 in male Fisher 344 rats of 64 mg/kg. Total hepatic fatty acid composition, as well as altered organ weights, including a reduction in heart weight, were observed following PFDA exposure (Olson and Andersen, 1983). We have previously reported that PFDA reduces resting heart rate, body temperature and serum thyroid hormones in rats over a delayed time-course (Langley and

Pilcher, 1985). The thyroid state can modulate heart mass (Ciaraldi and Marinetti, 1977) as well as cardiac adrenergic function (Gross and Lues, 1985). Based on reported observations that PFDA lowered resting heart rate in vivo, reduced heart weight, and reduced serum thyroid hormones in rats, studies were initiated to further define PFDA's effects on cardiac function. In the present work, the effect of PFDA on the functional responses of the isolated perfused rat heart to adrenergic stimuli was investigated. The functional parameters of heart rate (HR) and right ventricular pressure (RVP) were measured in response to 1) sympathetic nerve stimulation or 2) the direct infusion of norepinephrine. In addition, the effect of PFDA on the binding characteristics of myocardial beta receptors was investigated.

METHODS

MATERIALS. PFDA (Nonadecafluorodecanoic acid, 98%) was obtained in crystalline form from Aldrich Chemical Co. (Milwaukee, Wisconsin). The salts used in the Krebs-Hensleit perfusate were purchased from Fisher Scientific, Inc. (Cincinnati, Ohio). Propylene glycol, bovine serum albumin, l-norepinephrine HCl, l-isoproterenol HCl, and d,l propranolol HCl were obtained from Sigma Chemical Co. (St. Louis, Missouri). (-) [^{3}H]-Dihydroalprenolol, specific activity 90 Ci/mmole was purchased from New England Nuclear (Boston, Mass.).

ANIMALS. Male Wistar rats 175-225 g from Harlan Sprague-Dawley, Inc. (Indianapolis, Indiana) were housed separately in a temperature controlled room with an 11 hr/13 hr light-dark cycle. The rats were fed standard lab chow (Ralston-Purina Formulab® #5508) and given tap water ad libitum for a

period of 1 week prior to use. Dosing solution (75 mg PFDA/ml) was prepared in a propylene glycol-water vehicle (1:1) and administered in a volume of 1 ml/kg body weight. For the initial time-course study, groups of 4 to 6 rats were given a single dose of PFDA (75 mg/kg) between 1600 and 1700 hours, then sacrificed by decapitation 4,6,8, or 10 days after dosing for isolated heart experiments with sympathetic nerve stimulation. Serum was collected from these animals for thyroid hormone determinations. Body weights and food consumption were measured daily to the nearest gram. Weight-matched, pair-fed controls were vehicle-injected (ml/kg) and given the amount of food consumed daily by the corresponding PFDA-treated rats. Pair-fed controls were sacrificed 4,6,8, or 10 days after injection with vehicle, hearts were isolated and sera collected for thyroid hormone determinations. In addition, hearts were isolated and sera collected from a group of control rats allowed to feed ad libitum. Data on serum thyroid hormone levels were previously reported (Langley and Pilcher, 1985).

In the norepinephrine infusion experiment, hearts were isolated from 6 PFDA, 6 pair-fed or 6 control ad lib fed rats 8 days after dosing with PFDA (75 mg/kg) or vehicle (1 ml/kg).

Isolated, Perfused Heart Experiments

Hearts were prepared using a modification of the Langendorff procedure (Langley and Weiner, 1980). Following decapitation, hearts were rapidly exposed by removal of the sternum and perfused in situ via the ascending aorta at 7 ml/min with a modified Krebs/Henseleit solution containing: NaCl 118mM, $NaHCO_3$ 27.2 mM, KH_2PO_4 1mM, $MgSO_4$ 1.2 mM, KCl 4.8 mM, EDTA 0.5 mM, Dextrose 11.1 mM, $CaCl_2$ 2.5 mM, and 0.4% w/v BSA. The oxygen tension and pH of the perfusion solution were maintained by bubbling 95% O_2/5% CO_2 through

the solution. The temperature of the perfusate entering the heart was maintained at 32 $\pm$ 0.3°C by an MGW LAUDA Model T-1 constant temperature bath.

In experiments which involved nerve stimulation, both right and left stellate ganglia with intact sympathetic postganglionic nerve fibers to the heart were carefully isolated and dissected away from the surrounding tissue. The heart with intact sympathetic innervation was removed from the thorax and suspended via the aortic cannula. A PE-200 catheter connected to a Statham P-23 D pressure transducer was inserted into the right ventricle via the pulmonary trunk for right ventricular pressure (RVP) measurements. In addition, a three-lead electrogram recording with integrated heart rate (HR) was obtained from three platinum electrodes placed on the surface of the isolated heart and connected to a GOULD ECG-BIOTACH amplifier unit. RVP, HR, and the electrograms were simultaneously recorded using a 4-channel GOULD 2400S physiological recorder. The postganglionic nerve bundles were carefully suspended from the bipolar stimulation electrodes of a GRASS Model SD-9 stimulator. Stimulation of the nerves was applied with supra-maximal voltage (approximately 10 volts), a 2.0 msec impulse duration and a 0.02 msec delay. Stimulations were applied at various frequencies (0.2, 0.4, 0.8, 1.0, 2.0, 4.0, and 8.0 Hz) and stimulation periods (5.0, 2.5, 1.25, 1.0, 1.0, and 1.0 min) respectively, with a 10 min interval between each stimulation period.

In isolated heart experiments involving the direct infusion of norepinephrine (NE) hearts were perfused and suspended from the aortic cannula as described above without prior isolation of the sympathetic nerve fibers. Infusions were carried out using a 21 G 1½" syringe needle connected by PE-160 tubing to a 5 ml syringe mounted in a Harvard Apparatus

Compact Infusion pump which maintained a constant rate of drug infusion. Varying concentrations of norepinephrine (NE) were delivered to the heart by placing the syringe needle into the rubber tubing of the perfusion system near the aortic cannula and varying the infusion rate of a 5×10^{-5} M working solution of NE from the syringe pump. The working solution of NE was prepared prior to each experiment from a stock solution of NE (1×10^{-2} M) stabilized with 0.2 mM sodium metabisulfite. The flow of the syringe pump was less than or equal to 1% of the total perfusion flow rate. There were 10-15 min "wash-out" periods between each infusion period. Recordings of RVP, HR, and the electrograms were obtained as described above.

Pre-stimulation parameters were recorded immediately prior to each stimulation or infusion period. The maximum-stimulated values were the peak values of RVP or HR noted during the stimulation or infusion period.

Cardiac Membrane Preparation (30,000 x g fraction)

The method of Williams et al. (1977) was used to prepare "cardiac membrane fragments" for [^{3}H] dihydroalprenolol (^{3}H-DHA) binding. Pooled hearts were minced with scissors and homogenized in 10 volumes of ice-cold buffer (0.25 M sucrose, 5 mM TRIS-HCl pH 7.4, 1 mM $MgCl_2$) using a Brinkman Polytron PCU-2 homogenizer at 1/2 maximal speed for 15 sec. The homogenate was centrifuged at 480xg for 10 min at 4°C to remove cellular debris. The resulting supernatant was centrifuged at 30,000xg for 10 min at 4°C. The resulting pellet was washed by re-suspending in incubation buffer (50 mM TRIS-HCl, pH 7.5 and 10 mM $MgCl_2$) and re-centrifugation at 30,000xg for 10 min at 4°C. The final pellet was re-suspended in incubation buffer to yield a protein concentration of 3 to 5 mg/ml. Protein

content was determined by the method of Lowry et al. (1951). All centrifugation steps were performed in a Dupont Sorvall RC-5 Superspeed refrigerated centrifuge.

[^{3}H]-Dihydroalprenolol ([^{3}H]-DHA) Binding

Saturation binding experiments were carried out with aliquots from the resuspended 30,000xg pellet. The aliquots were incubated at 37°C for 10 min. in a volume of 200 ul containing 50 mM TRIS-HCl, pH 7.5, 10 mM $MgCl_2$, (-)[^{3}H]-DHA (0.1 to 4 nM) and 100 to 200 ug of membrane protein. Duplicate samples were run at each concentration of [^{3}H]-DHA in the presence and absence of 10 uM d,1-propranolol. The reaction was terminated by addition of 2 ml of ice-cold incubation buffer to each sample followed by rapid vacuum filtration through Whatman GF/C filters. The filters were washed with an additional 10 ml of cold incubation buffer and allowed to dry. Filters were placed in scintillation vials containing 4 ml of Aquasure® liquid scintillation cocktail (New England Nuclear, Boston, Mass.) and counted in a Packard PL Tri-Carb liquid scintillation counter. Specific binding to beta-adrenergic receptors was determined by substracting the [^{3}H]-DHA bound in the presence of 10 uM propranolol (non-specific binding) from that in the absence of propranolol (total binding). Specific binding was approximately 60-80% of total [^{3}H]-DHA binding. The amount of [^{3}H]-DHA bound was calculated from the specific activity of the [^{3}H]-DHA and expressed as femtomoles [^{3}H]-DHA bound per mg protein. The saturation binding data were analyzed by the method of Scatchard (1949).

Competition Binding Experiments with Adrenergic Agonists

In order to investigate beta-adrenoceptor agonist binding characteristics, a protocol similar to that used by Kent et al. (1980) was

employed. Aliquots of the cardiac membrane preparation (100 to 200 ug protein) were incubated in a volume of 200 ul containing 50 mM TRIS-HCl, pH 7.5, 10 mM $MgCl_2$, 2 nM (-) [^{3}H]-DHA, and varying concentrations of either isoproterenol (5 nM to 50 μM) or norepinephrine (50 nM to 500 μM). Incubations were for 10 min. at 37°C. The reaction was terminated with cold buffer and filtered through Whatman GF/C filters. Filters were counted as described above. Agonist binding was quantitated by determining the percent displacement of bound [^{3}H]-DHA at various concentrations of agonist. The percent agonist bound versus agonist concentration was analyzed with a non-linear least-squares curve fitting procedure (Statistical Analysis Systems Institute, Cary, North Carolina) using the general model for ligand-receptor binding described by Birdsall et al. 1980. The experimental binding data were iteratively fit using the model for one, two, or three classes of binding sites. The model yielding the lowest value of the mean squares of residuals provided the best fit for the data. The computer analysis provided estimates of the affinity state(s) and relative proportion of each binding site.

RESULTS

The Effects of PFDA on Cardiac Function

Isolated rat heart with sympathetic nerve stimulation: A time-course for cardiac effects of PFDA was established by isolating hearts 4, 6, 8, or 10 days after treatment. Stimulation of the sympathetic nerve bundles resulted in an increase in both heart rate (HR) and right ventricular pressure (RVP) in all treatment groups. The nerve-stimulated increase in heart rate (ΔHR) was enhanced in hearts from PFDA-treated rats. The

increases in ΔHR were significant at 4 days (0.4, 2.0, & 4.0 Hz), at 6 days (4.0Hz), and at 8 days (0.4, 1.0, 2.0 & 8.0 Hz). These changes were due primarily to a significant decline in the intrinsic resting HR (Table 1). The greatest reduction in resting HR was found 8 days after PFDA treatment. The maximum nerve-stimulated HR's were enhanced by PFDA only on day 4 post-exposure. The pre-stimulation measurements of HR did not appear to change significantly during the course of individual experiments or within any treatment group.

The greatest differences in nerve-stimulated right ventricular pressure, ΔRVP, occurred 8 days after a single dose of PFDA (Table 2). Treatment with PFDA resulted in significantly lower ΔRVP at the higher frequencies of stimulation (2.0, 4.0 & 8.0 Hz) making the ΔRVP response curve very shallow compared to controls. The ΔRVP at 8 Hz was 52% less in hearts isolated from PFDA-treated rats compared to hearts isolated from pair-fed control rats. PFDA's effect on ΔRVP at 8 days post-exposure was due to a decline in the maximum nerve-stimulated RVP rather than a change in pre-stimulation values of RVP. In addition, at 8 days post-exposure, the pre-stimulation RVP values were stable throughout the experimental period.

The Effects of PFDA on the Responses of the Isolated Rat Heart to Infused Norepinephrine: PFDA's effect on nerve-stimulated responses in the isolated rat hearts was greatest 8 days after treatment. Therefore, a response curve of isolated hearts to infusion of the adrenergic neurotransmitter norepinephrine was generated 8 days after a single dose of PFDA. Again, PFDA caused a significant reduction in resting (intrinsic) HR in the isolated heart preparation without affecting the maximum HR attained at each concentration of norepinephrine (Table 3). As a consequence the

ΔHR was enhanced by PFDA treatment especially at the higher concentrations of infused norepinephrine (5, 10, 25, and 50 uM). PFDA's effect on the RVP response to infused norepinephrine was similar to that seen in the nerve-stimulated isolated heart preparation (Table 2). The ΔRVP response to norepinephrine was reduced in the PFDA-treated group at the higher concentrations of norepinephrine (5, 10, 25, 50 uM). Again this effect was due to a reduction in the maximum RVP attained at nearly all concentrations of infused norepinephrine. The pre-stimulation RVP remained fairly stable throughout the experimental period in hearts from all three groups.

The Effect of PFDA On β-Adrenergic Receptor Binding Characteristics

A Scatchard plot of the saturation binding data (Fig. 1) indicated that PFDA lowered the maximum binding capacity (Bmax) without changing the affinity of the receptor for [3H]-DHA. The observed shift in Bmax in the PFDA-treated group was statistically significant compared to ad lib controls. The mean Bmax values in fmol [3H]-DHA bound/mg protein (± sem) were as follows: PFDA, 139.2 ± 27.1; pair-fed control, 178.0 ± 40.6; and control ad lib, 206.6 ± 16.4. The affinity of ^{3}H-DHA for binding sites was the same in all three groups. The mean dissociation constants (Kd) in nM (± sem's) were as follows: PFDA, 2.86 ± 0.50; pair-fed control, 2.48 ± 0.45; and control ad lib, 2.45 ± 0.39. These were similar to previously reported values (Williams and Lefkowitz, 1978; Stiles and Lefkowitz, 1981; and Winek and Bhalla, 1979).

In order to evaluate PFDA's effect on β-receptor agonist binding properties, the kinetics of agonist displacement of a single concentration of bound [^{3}H]-DHA (2 nM) was measured in the same cardiac membrane preparation. Either norephinephrine or isoproterenol was tested for its

ability to displace [^{3}H]-DHA. In each case, a two-site binding model provided the best mathematical estimates for the affinity states of the β-receptor (Table 4). Others have reported that a two site model provides the optimum fit for β-receptor agonist binding in the rat heart (Kent et al. 1980). PFDA did not alter either the affinity constants (Ka's) or the relative proportion of sites for either norepinephrine or isoproterenol binding. The observed kinetic parameters were similar to those reported by Hancock et al. (1979) and Stiles and Lefkowitz (1981) in rat heart.

DISCUSSION

We have previously reported a significant fall in total circulating thyroid hormone levels in sera from the rats used for the isolated heart experiments reported herein (Langley and Pilcher, 1985). In addition to decreases in serum thyroid hormone levels we observed hypothermia and bradycardia which are common manifestations of hypothyroidism (Ibbertson, 1979; Mazzaferri, 1980). Hypothyroidism has been shown to lower the number of β-receptors in rat hearts (McConnaughey et al. 1979; Stiles and Lefkowitz, 1981) without changing the binding affinity of the receptor (Stiles and Lefkowitz, 1981). In addition, the modulation of myocardial β-receptors by thyroid hormone is reflected in altered responsivity of the tissue (Brodde et al. 1980).

An examination of the time-course of myocardial effects of a single dose of PFDA showed that maximal changes in sympathetic nerve-stimulated HR and RVP occurred 8 days after treatment with PFDA. The effects of PFDA on the responses of the isolated heart were qualitatively similar when either sympathetic nerve stimulation or infusion of norepinephrine was employed to

stimulate cardiac activity. These results indicate that the effects are apparently mediated by an action on the myocardium rather than on the release of norepinephrine in response to stimulation of the sympathetic nerves.

PFDA caused the ΔHR and ΔRVP elicited by nerve stimulation or norepinephrine infusion in the isolated heart to deviate from control values in opposite directions. It must be emphasized that PFDA's effect on ΔHR was largely due to a reduction in intrinsic (resting) HR with no significant change in maximum HR whereas the reduced ΔRVP was the result of a lower maximum RVP. This effect of PFDA on resting HR in vitro is similar to the effect on resting HR in vivo previously reported (Langley and Pilcher, 1985). The fact that PFDA reduced resting HR in vitro as well as in vivo suggests that PFDA produces this effect by an action on the myocardium rather than via the autonomic nerves since the isolated heart lacks autonomic influences in the resting situation. Since diastolic depolarization in dominant pacemaker cells of the sinoatrial node (SA) node normally controls HR (Vassalle, 1982), our data suggest a PFDA-induced alteration in SA nodal function.

The effect of PFDA on the inotropic response of the isolated heart to norepinephrine was manifested in a lower ΔRVP due to a reduction in the maximum stimulated RVP. This reduction was most pronounced at higher rates of nerve stimulation or at higher infused concentrations of norepinephrine suggesting that the heart's ability to respond maximally was impaired by PFDA treatment.

The mechanism of enhancement of contractility by catecholamines involves a sequence of events initiated by stimulation of the β-adrenergic receptor on the surface of the myocardial cell which ultimately leads to an

enhancement of intracellular Ca++ availability (Katz, 1977). Responsiveness to catecholamines can be modulated at the membrane-receptor level by either a change in the number of receptors and/or an alteration of the affinity of the receptor for an agonist. PFDA reduced the apparent number of β-receptors without a significant change in the affinity of the receptor which may account for the decreased inotropic response observed with either nerve stimulation or NE infusion. The decrease in in vitro HR, reduced inotropic response of the heart to stimulation and reduced number of beta receptor binding sites reported herein may reflect the changes that we observed in serum thyroid hormone levels. These data tend to support our previous conclusion that some of the biological changes that occur several days after PFDA treatment may in part be secondary to early decreases in serum thyroid hormone levels (Langley and Pilcher, 1985).

Footnotes

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TABLE 3

Effect of Infused Norepinephrine on Heart Rate (beats/min) and Right Ventricular Pressure (mm Hg) in the Isolated Rat Heart. Values are Mean ± SEM.

	Treatment Group		Norepinephrine ($X10^{-7}M$) 0.05	0.10	0.25	0.5	1.0	2.5	5.0
	Control n=5	S[a]	39±2	51±6	49±7	59±4	62±3	62±5	59±5
		P[b]	37±1	35±2	29±2	26±2	25±3	20±4	29±7
		Δ	2±2	16±4	20±5	35±2	37±3	42±6	35±7
RVP	PFDA n=6	S	35±3	45±3[d]	49±2[d]	50±2[d]	48±3[d]	46±3[d]	46±3[d]
		P	26±2[d]	33±1[d]	34±1	33±2	29±2	26±2	25±3
		Δ	8±3	12±3	15±2	17±2[d]	19±2[d]	21±2[d]	21±3[d]
	Pair-fed Control n=6	S	45±4	55±1	57±2	60±3	63±3	62±4	62±3
		P	33±3	40±2	39±3	36±3	36±2	31±3	29±3
		Δ	11±3	16±3	18±2	24±2	27±2	32±2	33±2
	Control n=5	S	227±4	241±8	240±5	253±6	270±12	274±15	291±21
		P	223±4	226±8	228±8	224±5	224±5	227±5	224±7
		Δ	4±1	15±8	12±6	28±7	46±10	47±13	67±18
HR	PFDA n=6	S	190±7	200±11	194±8	207±16	225±14	241±12	250±10
		P	184±8[d]	190±11	172±8	170±7[d]	168±6[d]	167±9[d]	161±8[d]
		Δ	6±2	11±3	22±5	37±17	57±13	74±9[c]	89±9[c]
	Pair-fed Control n=6	S	221±7	215±6	211±6	212±8	221±8	224±8	234±6
		P	215±4	196±7	189±6	197±7	195±6	201±4	197±7
		Δ	6±3	18±6	22±7	15±9	27±6	23±7	37±9

[a] S = the maximum HR or RVP attained during the infusion period.
[b] P = the HR or RVP recorded immediately prior to the infusion period.
[c] Significantly greater than pair-fed control, $p<.05$, Student's t-test.
[d] Significantly less than pair-fed control, $p<.05$, Student's t-test.

TABLE 4

BINDING PARAMETERS OF β-ADRENERGIC RECEPTOR AGONISTS
ESTIMATED BY COMPETITIVE ANTAGONISM OF [^{3}H]-DHA BINDING

		SITE	Affinity Constant (Ka)[a]	Relative Proportion of Site
Norepinephrine	Control (Ad lib)	A	$6.66 \pm 1.30 \times 10^3$	.56
		B	$7.32 \pm 1.91 \times 10^5$	.44
	PFDA	A	$7.71 \pm 2.02 \times 10^3$	.50
		B	$1.50 \pm 0.41 \times 10^6$	.50
	Pair-fed Control	A	$3.56 \pm 1.25 \times 10^3$	.43
		B	$9.86 \pm 2.49 \times 10^5$	.57
Isoproterenol	Control (Ad lib)	A	$5.58 \pm 1.33 \times 10^5$	.55
		B	$1.22 \pm 0.65 \times 10^7$	.45
	PFDA	A	$3.49 \pm 0.90 \times 10^5$	.56
		B	$3.23 \pm 0.99 \times 10^7$	.44
	Pair-fed Control	A	$3.25 \pm 0.76 \times 10^5$	.47
		B	$2.92 \pm 0.55 \times 10^7$	.53

[a] The estimates of Ka are expressed as mean ± asymptotic standard error.

TABLE 1

Effect of Sympathetic Nerve Stimulation on Heart Rate (beats/min) in the Isolated Rat Heart. Values are Mean ± SEM.

	DAYS AFTER TREATMENT		Stimulation Frequency (Hz) 0.2	0.4	0.8	1.0	2.0	4.0	8.0
AD LIBITUM CONTROL		S[a]	247±3	262±4	291±12	291±10	317±15	342±11	343±15
		P[b]	232±3	228±6	235±5	231±5	227±6	236±6	245±4
	n=5	Δ	16±6	34±7	56±13	60±13	89±14	106±12	98±13
		S	257±13	283±12[c]	276±23	275±25	302±17[c]	318±12[c]	322±17
	4	P	204±8	224±11	214±11	209±15	206±7	207±5	213±10
	n=4	Δ	53±6	59±7[c]	62±18	64±13	97±11[c]	112±8[c]	109±9
		S	259±23	259±16	272±21	279±19	311±19	330±15	322±17
	6	P	205±8	200±7[d]	191±6[d]	194±7	192±6[d]	191±7[d]	191±7[d]
	n=6	Δ	54±20	59±16	82±19	85±18	119±18	139±17[c]	113±19
PFDA									
		S	202±13[d]	241±16	257±19	263±19	287±23	301±23	315±19
	8	P	169±10[d]	178±6[d]	187±16	181±15[d]	169±7[d]	179±11[d]	171±7[d]
	n=6	Δ	33±7	63±11[c]	70±14	82±14[c]	119±16[c]	125±16	144±13[c]
		S	222±13	236±30	249±26	255±25	292±16	305±23	322±23
	10	P	185±6	181±16	174±10	172±11	177±9	172±6	183±6
	n=3	Δ	37±9	55±17	75±21	83±24	115±23	133±28	139±27
		S	238±6	234±4	250±6	244±2	255±7	265±12	292±15
	4	P	207±5	218±5	212±6	206±7	210±9	207±10	209±7
	n=4	Δ	31±6	16±6	37±7	39±8	46±14	58±15	83±21
		S	237±12	242±10	257±16	266±15	289±15	309±13	316±14
	6	P	229±10	218±9	217±10	222±9	220±10	222±12	232±10
	n=6	Δ	8±3	25±9	41±8	44±10	69±15	87±9	87±11
PAIR-FED CONTROL		S	250±11	258±11	262±10	265±9	280±10	306±9	309±12
	8	P	226±8	226±9	223±10	226±12	223±10	219±8	222±12
	n=6	Δ	24±3	32±5	40±7	39±5	57±9	87±9	87±15
		S	245±14	258±25	275±47	253±17	282±3	310±0	312±6
	10	P	223±24	232±12	225±29	203±28	210±21	193±30	203±7
	n=3	Δ	22±18	26±18	50±22	49±21	72±18	117±30	109±12

[a] S = the maximum HR attained during the stimulation period.
[b] P = the resting (prestimulation) HR recorded immediately prior to the stimulation period.
[c] Significantly greater than pair-fed control, $p < .05$, Student's t-test.
[d] Significantly less than pair-fed control, $p < .05$, Student's t-test.

TABLE 2

Effect of Sympathetic Nerve Stimulation on Right Ventricular Pressure (mm Hg) in the Isolated Rat Heart. Values are Mean ± SEM.

	DAYS AFTER TREATMENT		Stimulation Frequency (Hz) 0.2	0.4	0.8	1.0	2.0	4.0	8.0
AD LIBITUM CONTROL		S[a]	37±2	38±1	41±3	39±2	43±3	46±3	47±3
		P[b]	34±1	32±2	31±2	30±3	28±2	27±2	27±2
	n=5	Δ	3±1	5±1	10±1	9±2	15±2	19±1	20±1
		S	28±3	29±5	31±7	31±7	36±8	40±7	42±8
	4	P	26±3	20±2	20±2	20±2	20±2	18±3	17±3[c]
	n=4	Δ	2±2	9±3	11±5	11±5	17±5	22±6	25±7
		S	36±2	42±2	43±2	43±2	46±2	49±4	46±3[c]
	6	P	29±3	31±3	31±2	29±2	30±3	31±2	31±3
	n=6	Δ	8±2	11±2	13±2	15±2	16±2	18±4	17±4
PFDA									
		S	30±3	31±3	33±3	33±3	34±3	34±3	36±4
	8	P	26±3	28±3	29±3	28±3	27±3	27±3	26±3
	n=6	Δ	4±1	4±1	5±1	6±2	7±2[c]	7±2[c]	10±3[c]
		S	29±4	32±2	34±3	35±3	37±3	35±3	35±2[c]
	10	P	25±4	26±3	24±4	24±4	23±4	23±4	20±2
	n=3	Δ	4±1	8±0	10±1	11±1	14±1	12±2	15±1
		S	36±6	37±7	41±8	43±8	43±8	48±8	51±8
	4	P	29±4	28±4	26±3	25±2	24±2	23±2	24±2
	n=4	Δ	7±2	9±2	15±4	18±5	19±5	25±6	27±6
		S	36±2	37±2	41±2	43±2	45±2	52±2	56±2
	6	P	31±3	32±1	31±1	29±2	31±1	27±2	27±2
	n=6	Δ	5±2	5±1	10±2	14±2	15±2	23±3	29±2
PAIR-FED CONTROL		S	35±3	36±2	38±4	38±3	41±4	44±4	47±4
	8	P	32±3	32±3	30±3	28±3	27±4	27±4	26±3
	n=6	Δ	4±1	4±1	8±1	9±1	14±2	17±2	21±2
		S	32±3	34±4	38±3	38±4	42±2	44±2	45±2
	10	P	28±3	29±4	29±3	28±4	29±3	29±2	28±3
	n=3	Δ	4±1	5±1	9±1	10±1	13±3	15±3	17±4

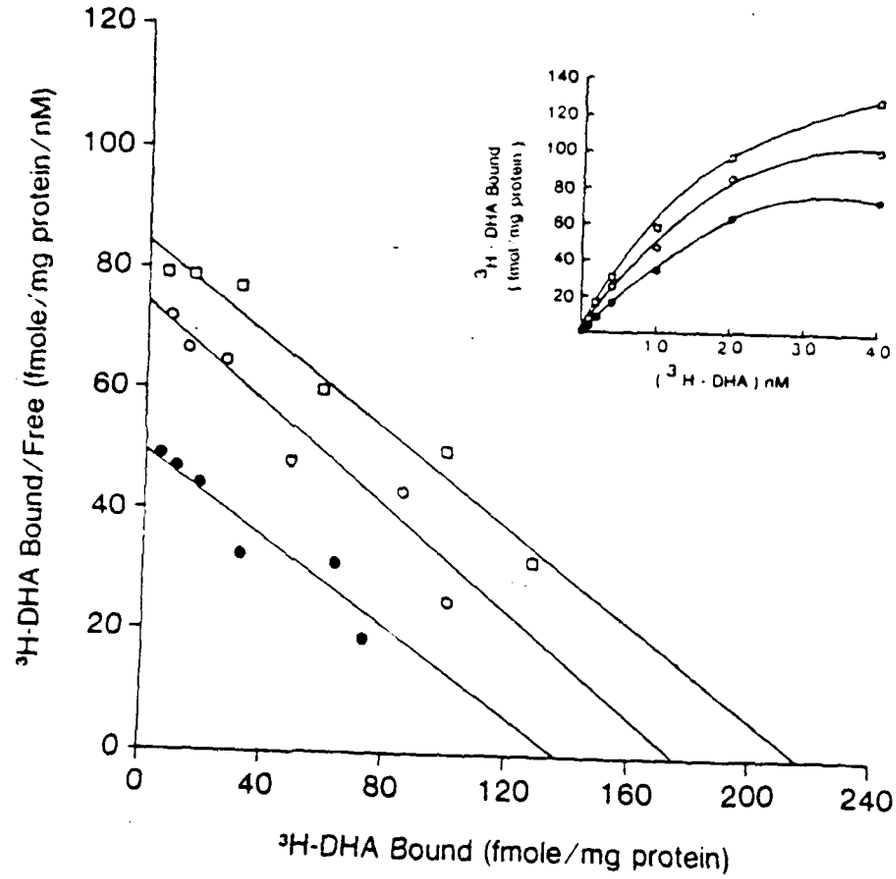
[a] S = the maximum RVP attained during the stimulation period.

[b] P = the RVP recorded immediately prior to the stimulation period.

[c] Significantly less than pair-fed control, $p < .05$, Student's t-test.

FIGURE 1

SATURATION BINDING ISOTHERM WITH SCATCHARD PLOT OF [^{3}H]-DHA BINDING IN CARDIAC MEMBRANE FRAGMENTS FROM PFDA (●), n=4, PAIR-FED (O), n=4 AND CONTROL AD LIB (□), n=4 RATS 8 DAYS POST-EXPOSURE. Saturation binding was determined over a range of [^{3}H]-DHA concentrations from 0.1 to 4nM. The lines for PFDA (r=0.98), pair-fed control (r=0.95), and control ad lib (r=0.98) in the Scathchard plot were determined by linear regression analysis. Estimates of the dissociation constant (Kd) and maximum binding capacity (Bmax) for each experiment were obtained from -1/slope and the x-intercept respectively. Differences between PFDA-treated and control groups were determined using Student's t-test, $p<0.05$.


120
100
80
60
40
20
0
0
40
80
120
160
200
240
³H-DHA Bound/Free (fmole/mg protein/nM)
³H-DHA Bound (fmole/mg protein)
140
120
100
80
60
40
20
10
20
30
40
(³H - DHA) nM

MANUSCRIPT

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The Effects of Perfluoro-n-decanoic acid (PFDA) on rat heart beta receptors and adenylate cyclase.

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Short Title

PFDA, adenylate cyclase and beta receptors.

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Abstract

The Effects of Perfluoro-n-decanoic acid (PFDA) on rat heart beta receptors and adenylate cyclase. Pilcher, Gary D. and Langley, Albert E. (1986) Tox. and Appl. Pharm.

Perfluoro-n-decanoic acid (PFDA) is a member of a family of compounds with numerous industrial applications. Recent reports have indicated that the effects of PFDA may involve an action on the structure of biological membranes which results in an alteration of function. In the present study we extended our work on the membrane actions of PFDA by examining its effects on myocardial beta adrenoceptor binding characteristics and adenylate cyclase. Eight days following a single injection of PFDA the apparent number of beta receptor binding sites was reduced compared to pair-fed controls. This change in beta receptor binding capacity was reflected in a reduced ability of norepinephrine to activate adenylate cyclase. No alterations were observed in basal adenylate cyclase activity or in the ability of NaF or GppNHp to stimulate the enzyme. The effects of PFDA on certain components of the membrane lipid bilayer are discussed.

INTRODUCTION

Perfluoro-n-decanoic acid (PFDA) or nonadecafluorodecanoic acid is a 10 carbon straight-chain acid, $C_{10}F_{19}O_2H$, having complete substitution of fluorine for hydrogen in the aliphatic portion of the molecule. Perfluoroalkanoic acids and structurally related compounds are used widely in manufacturing as anti-wetting agents (Guenthner and Victor, 1962) and in the treatment of fabric and paper (Bryce, 1964).

In rats a single dose of PFDA near the LD_{50} has been reported to produce hypophasia and severe weight loss (Olsen and Andersen, 1983, Langley and Pilcher, 1985) bradycardia, hypothermia and decreased circulating levels of thyroid hormones (Langley and Pilcher, 1985). We previously reported reduced responsiveness of isolated rat hearts to adrenergic stimulation and a decrease in the apparent number of β-receptor binding sites in hearts from PFDA-treated rats (Pilcher and Langley, 1986).

Several studies have indicated that PFDA alters lipid metabolism, specifically causing major shifts in hepatic fatty acid composition (Olson and Andersen, 1983; George and Andersen, 1986) as well as tissue cholesterol

levels (George and Andersen, 1986) and significant induction of peroxisomal fatty acid oxidation (Harrison et al. 1986). Other reports have suggested that exposure to PFDA results in altered plasma membrane function either via direct cellular exposure *in vitro* (Rogers et al. 1982) or in red blood cells following *in vivo* exposure (Olson et al. 1983). In the present study the effect of PFDA on the biochemical transducer of adrenergic receptor mediated activation of the myocardium, adenylate cyclase was investigated. In addition, beta receptor binding characteristics were examined in the same fraction and correlated with changes in adenylate cyclase.

METHODS

Male Wistar rats weighing 175-225 g were obtained from Harlan Sprague-Dawley Inc., Indianapolis, Indiana. Rats were housed separately on wood chip bedding in a temperature-controlled room (22.8 $\pm$ 2.7°C) with an 11 hr./13 hr. light/dark cycle. The rats were fed a standard rat chow, Ralston Purina Formulab #5508®, and given tap water ad libitum until they were used, typically 5-7 days after arrival.

In all experiments, PFDA treatment consisted of ip. administration of PFDA (75 mg/ml) dissolved in a propylene glycol-water (1:1) vehicle delivered in a volume of 1 ml/kg body weight. Weight-matched pair-fed controls were given the same vehicle in a volume of 1 ml/kg body weight and then pair-fed to the daily food consumption record of the appropriate PFDA-treated animals. In all experiments, rats were given a single dose of PFDA or vehicle and sacrificed 8 days after injection for collection of heart tissue. Ad libitum controls were untreated and allowed to eat and drink ad libitum.

Beta receptor binding and adenylate cyclase activity were measured in a crude membrane fraction prepared by the method of Drummond and Severson (1974). Rats were decapitated and their hearts rapidly removed, and chilled in ice-cold homogenizing buffer. The hearts were blotted, weighed, trimmed of excess connective tissue, minced with scissors and homogenized in 10 volumes of fresh homogenizing buffer at 4°C (0.25 M Sucrose, 25 mM TRIS-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 0.5 mM (EGTA)) with a Brinkman Polytron PCU-2 at 1/2 maximum speed for 20 sec. The crude homogenate was passed through 4-ply cheesecloth to remove additional

connective tissue, then centrifuged at 1000xg for 15 min at 4°C. The resulting pellet was washed twice by resuspending in the original volume of homogenizing buffer and re-centrifuged at 1000xg. The final pellet was resuspended in 10 volumes of incubation buffer (25 mM TRIS-HCl, pH 7.5, containing 5 mM $MgCl_2$, 2mM DTT, and 0.5 mM EGTA) yielding a protein concentration of 5 to 10mg/ml. Proteins were determined by the method of Lowry et al. (1951). All centrifugation steps were performed in a DuPont Sorvall RC-5 Superspeed refrigerated centrifuge.

[^{3}H]-Dihydroalprenolol ([^{3}H]-DHA) Binding

Saturation binding experiments were carried out with aliquots of the resuspended 1000xg pellet using a modification of the method of Williams, et al. (1976). The aliquots were incubated at 37°C for 10 min. in a volume of 22 ul containing 50 mM TRIS-HCl, pH 7.5, 10 mM $MgCl_2$, (-)[^{3}H]-DHA (0.03 to 4 nM) and 100 to 500 ug of membrane protein. Duplicate samples were run at each concentration of [^{3}H]-DHA in the presence and absence of 10 uM (±) propranolol. The reaction was terminated by addition of 2 ml of ice-cold incubation buffer to each sample followed by rapid vacuum filtration

through Whatman GF/C filters. The filters were washed with an additional 10 ml of cold incubation buffer and allowed to dry. Filters were placed in scintillation vials containing 4 ml of Aquassure® liquid scintillation cocktail and counted on a Beckman LS 7800 liquid scintillation counter. Specific binding was determined by subtracting the [^{3}H]-DHA bound in the presence of 10 uM (±) propranolol (non-specific binding) from that in the absence of propranolol (total binding). Specific binding was approximately 50% of total [^{3}H]-DHA binding. The saturation binding data were analyzed by the method of Scatchard (1949).

Adenylate Cyclase Assay

Adenylate cyclase activity was assayed using the method of Salomon, et al., (1974). The incubation mixture contained 25 mM TRIS-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM creatine phosphate, 100 units creatine phosphokinase/ml, 1 mM cyclic adenosine monophosphate (cAMP), 1 mM 3-isobutyl-1-methyl-xanthine, 2 mM DTT, 0.5 mM EGTA, 0.02% ascorbic acid, 20 uM guanosine triphosphate (GTP), and 1 mM adenosine triphosphate (ATP) containing at least 1-2

microcuries (u Ci) [α-^{32}P]ATP in a total volume of 100 ul. When the non-hydrolyzable GTP analogue guanylyl imidodiphosphate (GppNHp) was included in the incubation mixture, 10 uM GppNHp was substituted for GTP. Varying concentrations of norepinephrine or 10 mM NaF were tested for their ability to stimulate adenylate cyclase. The reaction was initiated by addition of the heart homogenate, run for 10 min at 30°C unless otherwise indicated, and terminated by addition of 10 ul of a "stop" solution containing 2% sodium dodecyl sulfate (SDS), 40 mM ATP, and 1 mM cAMP at pH 7.5. Approximately 10-20,000 CPM of [^{3}H]-cAMP was added in 60 ul to monitor recovery in the subsequent column chromatography steps. Each sample was placed in a boiling water bath for 3 min to completely solubilize membrane protein, 1 ml water was added to each tube and the tubes were vortexed. Blanks containing aliquots of homogenate, the incubation mixture plus the "stop" solution, were carried through the procedure in an identical manner.

The cAMP formed in each sample was isolated by sequential column chromatography first on Dowex 50 AG WX4 resin, and then on neutral alumina. Approximately 14.6 ml of liquid scintillation cocktail (Fisher-Scintiverse

II) was added to each sample eluate, mixed, and counted in a Beckman LS 7800 liquid scintillation counter. Average recovery of cAMP was approximately 70%. The procedure used to calculate enzyme activity was described by Salomon (1979). Enzyme activity was expressed as picomoles cAMP formed per 10 minutes per mg protein.

Thermodynamic Analysis of Basal Adenylate Cyclase

The temperature dependence of adenylate cyclase was examined using the method of Chatelain, et al., (1982). A 250 ul aliquot of adenylate cyclase incubation medium (described above) was allowed to equilibrate at the appropriate temperature for 1 min and the reaction initiated by the addition of 50 ul of the heart homogenate. Aliquots of 50 ul were removed from the mixture after 2, 4, 6, 8, and 10 min, and rapidly placed in tubes containing 100 ul of "stop solution" (described above). The samples were then subjected to sequential column chromatography on Dowex/Alumina, and counted as described above. Incubations were performed at 15°C, 20°C, 24°C, 26.5°C, 29°C, 30°C, 31.5°C, 33°C, 35°C, and 37°C. At temperatures of 24°C and

below, aliquots of the incubation mixture were removed at 2, 5, 10, 15, and 20 min following the addition of membrane preparation. The reactions were found to be linear with time, and a rate constant k_r was determined from the slope of the line when activity (p moles cAMP formed/mg protein) was plotted against time at each temperature. An energy of activation for adenylate cyclase was derived from the slope of each plot using the Arrhenius equation.

Materials

PFDA (Nonadecafluorodecanoic acid, 98%) was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). (-) [^{3}H]-dihydroalprenolol, sp. act. 33 Ci/mmole, and [α-^{32}P]-adenosine 5'- triphosphate, sp act. 90 Ci/mmole were purchased from New England Nuclear (Boston, Massachusetts. The [2,8 - ^{3}H]-adenosine 3',5'-cyclic monophosphate, sp. act. 15-30 Ci/mmole was obtained from ICN radioisotope division (Irvine, California). All other chemicals were obtained in high purity from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Binding of ^{3}H-DHA

A Scatchard plot of the saturation binding of [^{3}H]-DHA indicated a decrease in the maximum number of binding sites with no change in affinity (Figure 1). The mean Bmax value for the PFDA-treated group (36.0 $\pm$ 7.0 fmol/mg) was significantly lower than pair-fed controls (63.0 $\pm$ 5.8 fmol/mg) with no significant change in mean Kd values (PFDA, 1.63 $\pm$ 0.34 nM; and pair-fed controls, 2.13 $\pm$ 0.46 nM).

The Effect of PFDA on Adenylate Cyclase Activity

Adenylate cyclase activity was determined in the 1000xg fraction. This preparation yielded approximately 68% of the adenylate cyclase activity measured in the crude homogenate. The reduced number of β-receptors following PFDA treatment appeared to be reflected in a decrease in β-receptor-stimulated adenylate cyclase activity. PFDA reduced the ability of norepinephrine (1, 10 and 100 uM) to activate adenylate cyclase (Fig. 2). However, basal activity and activity in the presence of 10 mM sodium fluoride, or the non-hydrolyzable guanine nucleotide analog, guanylyl

imidodiphosphate (GppNHp), were not significantly affected by PFDA treatment (Table 1). In addition, the activity of adenylate cyclase in the presence of a high concentration of norepinephrine (1 mM) plus GppNHp was not significantly affected by PFDA treatment (Table 1).

Thermodependence of Basal Adenylate Cyclase Activity

An alteration in the membrane environment of adenylate cyclase might be reflected by changes in the thermodynamic properties of the enzyme. Adenylate cyclase in preparations from both PFDA and pair-fed controls exhibited discontinuities in their respective Arrhenius plots which are near the reported phase transition temperature of the plasma membrane in rat heart, 31°C (Gordon et al., 1978) (Fig. 4). The discontinuities for each group were PFDA, 30.9 $\pm$ 0.1°C; pair-fed controls, 30.2 $\pm$ 0.7°C; and control ad lib, 27.9 $\pm$ 1.9°C. Compared with pair-fed controls PFDA did not affect the calculated energies of activation (Ea) either above or below the phase transition point. However, both the PFDA-treated and pair-fed control groups exhibited significantly higher Ea's above the phase transition point compared to controls fed ad libitum. The mean energies of activation (kcal/mol) at temperatures above (Ea 1) and below (Ea 2) the phase

transition temperatures were: PFDA, Ea 1 = -12.81 ± 3.11, Ea 2 = -15.50 ± 1.84; pair-fed control, Ea 1 = -13.57 ± 1.51, Ea 2 = -18.91 ± 2.17; and control ad lib, Ea 1 = -5.02 ± 1.15, Ea 2 = -20.46 ± 2.39. These data suggest that the reduction in food consumption elevated the Ea for basal adenylate cyclase activity at temperatures above the breakpoint temperature.

DISCUSSION

The results of the binding experiments are similar to those previously reported in a 30,000xg cardiac preparation (Pilcher and Langley, 1986). PFDA significantly reduced the apparent number of β-receptors without a significant change in the affinity of the receptor. The kinetic constants of dissociation (Kd's) derived by Scatchard analysis of [^{3}H]-DHA binding were similar to previously reported values (Williams and Lefkowitz, 1978; Stiles and Lefkowitz, 1981; and Winek and Bhalla, 1979).

Stimulation of cardiac β-receptors results in activation of adenylate cyclase and increased cAMP formation (Sutherland, et al, 1965). A reduction in β-receptor density could lead to a decrease in the activation of adenylate cyclase by catecholamines. The PFDA-induced decrease in β-receptor number was reflected in a reduced ability of norepinephrine to

activate adenylate cyclase. The reduction was greatest at 10 uM norepinephrine which was near the ED50 value for adenylate cyclase activation. In this preparation no reduction in basal, NaF, or GppNHp stimulated adenylate cyclase was apparent suggesting that neither the guanine nucleotide regulatory subunit, nor the catalytic subunit of adenylate cyclase which are components of the inner leaflet of the bilipid membrane were affected by PFDA treatment.

PFDA did not alter the apparent phase transition temperature or energies of activation (Ea) for basal adenylate cyclase compared to pair-fed controls. On the other hand PFDA treatment and pair-feeding had a significant effect on the Arrhenius activation energy above the phase transition temperature when compared to controls fed ad libitum. Energies of activation were comparable to those reported by Chatelain et al. (1982) in rat sarcolemma and a similar phase transition temperature in rat heart sarcolemma was reported by Gordon et al. (1978). Basal and guanine nucleotide/fluoride stimulated adenylate cyclase activities are reportedly influenced by the lipid environment of the inner leaflet of the membrane bilayer (Houslay and Gordon, 1983). Thus, PFDA treatment did not

seem to significantly alter the physical characteristics of the immediate environment of that portion of adenylate cyclase which is located in the inner half of the bilayer. Indeed, this is consistent with the observation that PFDA lowers the β-receptor activation of adenylate cyclase and the subsequent inotropic effect without altering basal or fluoride-stimulated adenylate cyclase activity.

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FIGURE 1

SATURATION BINDING ISOTHERM WITH SCATCHARD PLOT OF [3H]-DHA BINDING FROM PFDA (●), n=4 AND PAIR-FED (0), n=4 RAT HEARTS 8 DAYS POST-EXPOSURE. Saturation binding was determined over the range of [3H]-DHA concentrations from 0.2 to 4nM. The lines for PFDA (r=0.98) and pair-fed control (r=0.99) in the Scatchard plot were determined by linear regression analysis. Differences between PFDA-treated and pair-fed control groups were determined using Student's t-test, $p<0.5$.

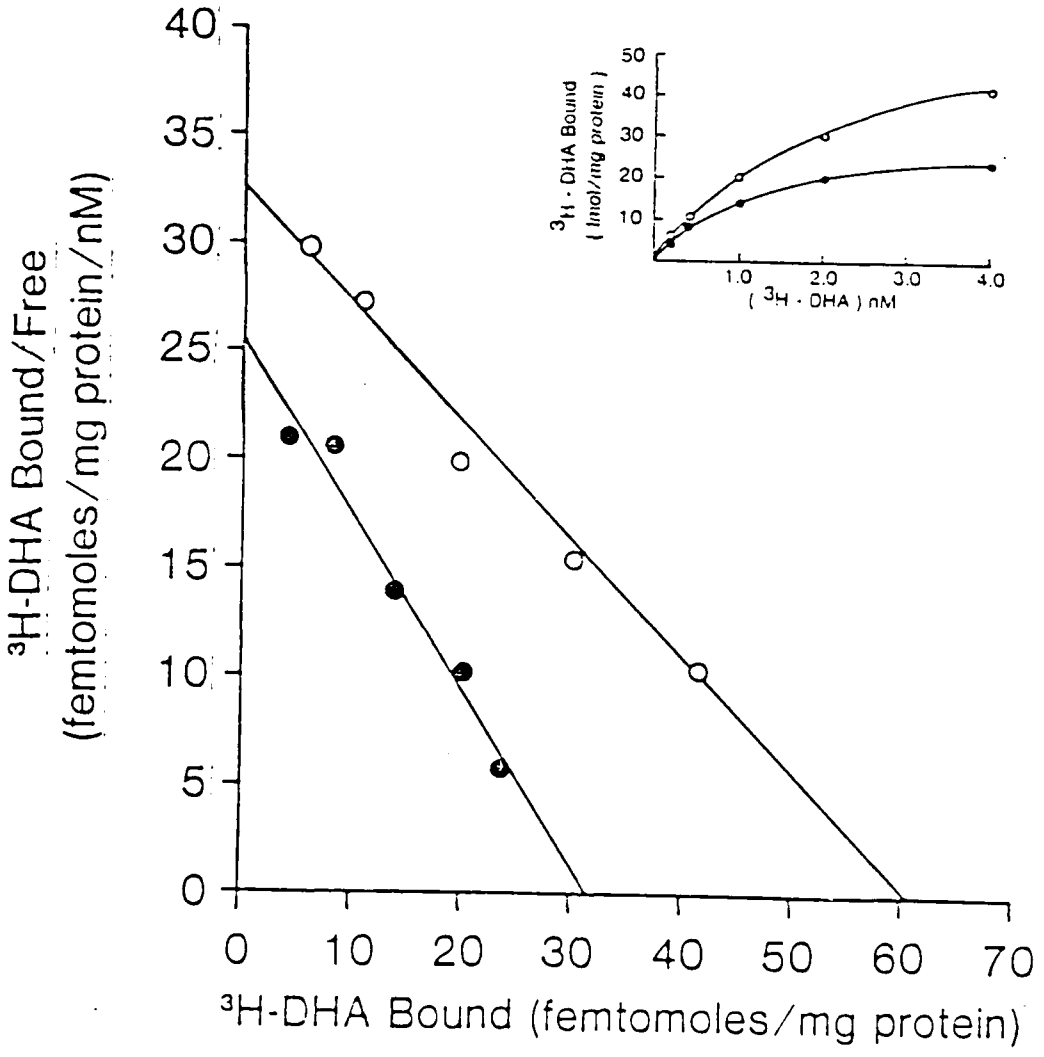
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
³H-DHA Bound/Free
(femtomoles/mg protein/nM)
³H-DHA Bound (femtomoles/mg protein)
50
40
30
20
10
1.0
2.0
3.0
4.0
(³H - DHA) nM

FIGURE 2

THE EFFECT OF PFDA (●, n=4) AND PAIR FEEDING (O, n=4) ON NOREPINEPHRINE-ACTIVATED ADENYLATE CYCLASE ACTIVITY. Each point represents the mean ± sem. * significantly less than pair-fed controls, $p<.05$, Student's t-test. Ad lib controls □, n=5

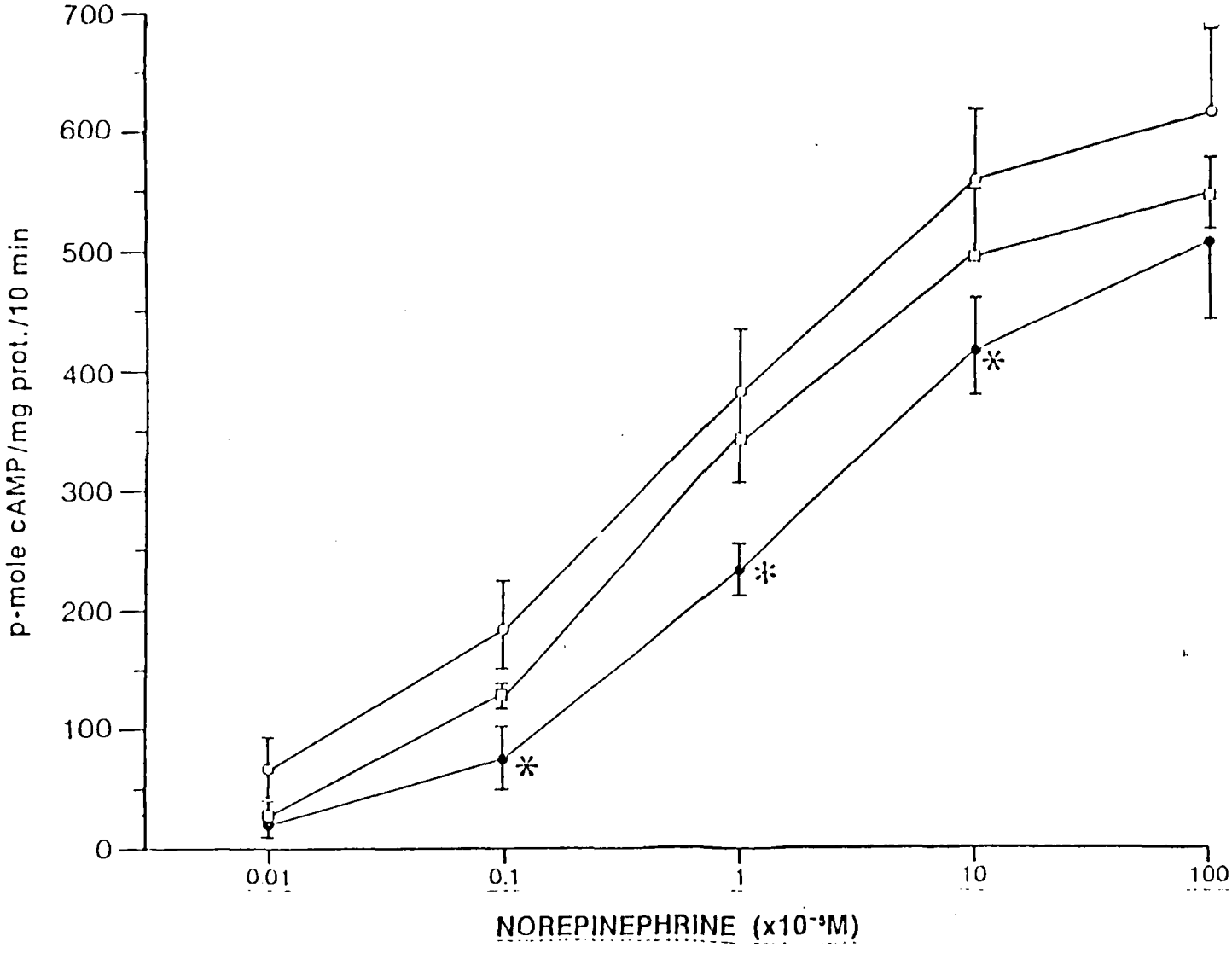
700
600
500
400
300
200
100
0
p-mole cAMP/mg prot./10 min
0.01
0.1
1
10
100
NOREPINEPHRINE (x10⁻⁹M)
*
*
*

FIGURE 3

ARRHENIUS PLOTS OF BASAL ADENYLATE CYCLASE ACTIVITY. Each point represents the mean of 2 to 4 determinations. Plots were constructed according to the arrhenius equation. In each group a two-segment line model provided a significantly better fit than a straight line model. Experiments were analyzed individually to obtain estimates of activation energies above (E_{act} 1) and below the break-point temperature (E_{act} 2). In addition, the break-point temperature (arrow) was determined in individual experiments. Differences between treatment groups were determined using ANOVA, $p<.05$, with Duncan's Multiple Range Test on the means.

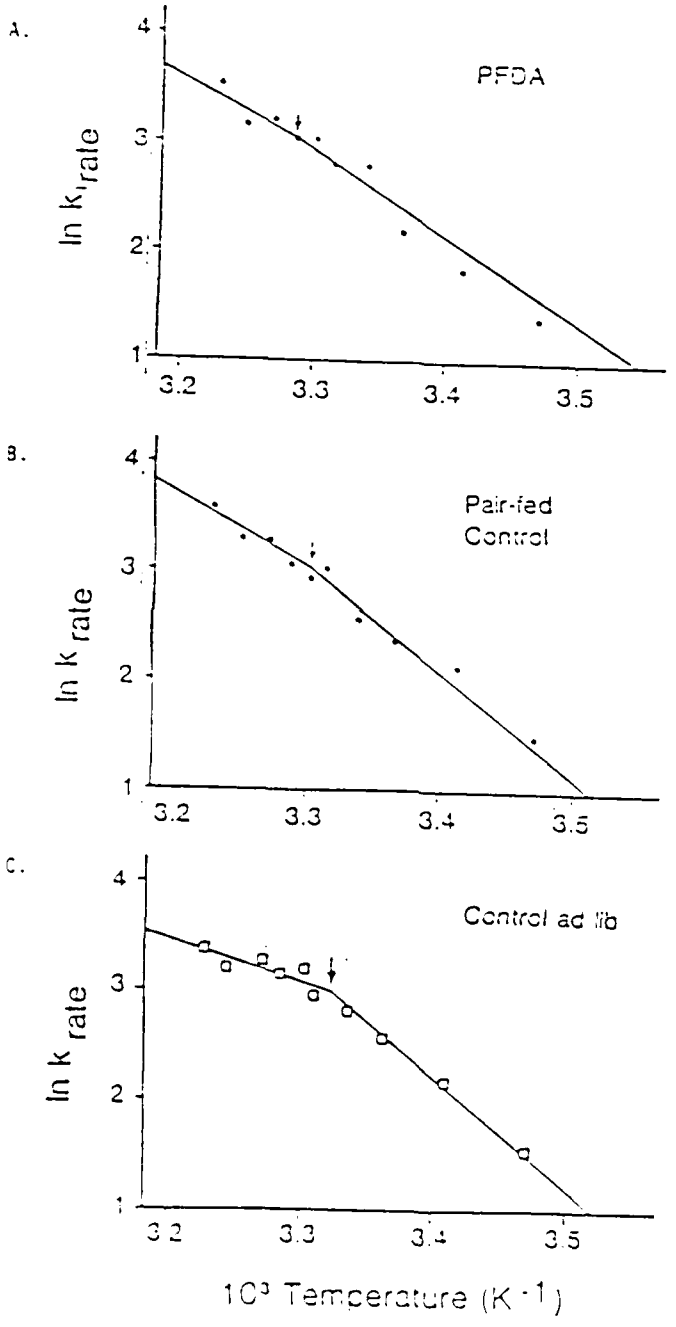
A.
PFDA
ln k rate
4
3
2
1
3.2
3.3
3.4
3.5
B.
Pair-fed
Control
ln k rate
4
3
2
1
3.2
3.3
3.4
3.5
C.
Control ad lib
ln k rate
4
3
2
1
3.2
3.3
3.4
3.5
10³ Temperature (K⁻¹)

TABLE 1

Adenylate Cyclase Activity

(pmol cAMP formed/mg protein/10 min)

	BASAL $\bar{X}\pm$SEM	10mM NaF[b] $\bar{X}\pm$SEM	GppNHp[a,b] $\bar{X}\pm$SEM	1mM NE + GppNHp[a,b] $\bar{X}\pm$SEM
PFDA (n=4)	300 ± 37	1029 ± 117	225 ± 22	870 ± 24
Pair-fed Control (n=4)	301 ± 28	1304 ± 155	330 ± 20	912 ± 79
Control ad lib (n=5)	267 ± 18	758 ± 54	314 ± 32	754 ± 45

[a] GppNHp = Guanylylimidodiphosphate, 10μM

[b] Activities expressed with basal activity subtracted

END

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DTIC