The Effect of Acute Dyflos (DFP) Treatment on [³H]Nicotine Binding to Mouse Brain Homogenate

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Abstract–One possible adaptive mechanism that might arise due to inhibition of cholinesterase in the brain is a down regulation of central cholinergic receptors. Therefore studies were performed to determine the effect of acute dyflos exposure on [³H]nicotine binding. Specific [³H] nicotine binding was demonstrated to be saturable, reversible, stereospecific, and inhibited by a number of nicotinic compounds. Scatchard analysis of specific [³H]nicotine binding produced a curvilinear plot that was resolved into high- and low-affinity sites with K_d values of $6 \cdot 1 \pm 2 \cdot 5$ and 114 ± 13 nM, and B_{max} values of $11 \cdot 8 \pm 3 \cdot 5$ and 182 ± 24 fmol (mg protein)$^{-1}$, respectively. The nicotinic binding sites in brain homogenate from dyflos-treated mice that were killed 20 min or 10 h after exposure did not exhibit significant alterations in binding parameters from control mouse brain homogenate. However, brain homogenate from treated mice that were killed 24 h after exposure resulted in statistically significant differences in the low-affinity K_D and B_{max} values from controls. Since no alterations were found in the high-affinity binding parameters and dyflos had only a minimal effect on the low-affinity site at 24 h, it was concluded that nicotinic receptor down regulation does not appear to be the mechanism through which the mouse functionally adapts to cholinesterase inhibition caused by acute dyflos treatment.

Diisopropylfluorophosphate (DFP, dyflos, isofluorophate) is a member of a class of organophosphate compounds that are all known to irreversibly inhibit acetylcholinesterase (Koelle & Gilman 1949; Holmstedt 1959). Dyflos inhibits acetylcholinesterase at both peripheral and central sites (Koelle & Gilman 1946; Mayer & Michalek 1971), with brain cholinesterase activity depressed in the mouse and rat for at least one month after a single treatment (Glow et al 1966; Kozar et al 1976; Martin 1985). There is evidence that some of the centrally mediated actions of dyflos are not the result of this central cholinesterase inhibition. The time course of dyflos-induced suppression of consummatory behaviour (Russell et al 1969), operant behaviour (Russell et al 1969; Overstreet et al 1974), spontaneous activity (Martin 1985) and motor coordination (Scimeca et al 1985) was not found to correlate with the time course of cholinesterase inhibition in the CNS. The finding that some centrally-mediated functions recovered before cholinesterase activity returned to baseline levels suggests that other adaptational factors may be important in determining the recovery of these functions following acute treatment with dyflos.

One possible mechanism that may account for a central adaptation to dyflos-induced cholinesterase inhibition is a down regulation of cholinergic receptors. Although the effect of dyflos treatment on the central muscarinic receptor has been studied (Schiller 1979; Ehlert et al 1980; Sivam et al 1983; Lim et al 1986), in mice (Uchida et al 1979) and in guinea-pigs (Yamada et al 1983), the effect of dyflos treatment on central nicotinic receptors has received much less investigation. Schwartz & Kellar (1983, 1985) have shown a reduction in the binding of [³H]acetylcholine to central nicotinic receptors after repeated treatment with dyflos; however, studies of its acute effect on these receptors are still lacking. Therefore, the present study was undertaken

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to determine if central nicotinic cholinergic receptors respond to acute treatment with dyflos and consequently exist as a possible adaptational mechanism to dyflos-induced cholinesterase inhibition. Specifically, the objective of this study was to determine the effect of a single exposure to dyflos vapour on the in-vitro binding of [³H]nicotine to mouse brain. Inhalation was chosen since it is the route most relevant to human dyflos intoxication. In addition, some of the previous studies from our laboratory, in which the adaptational processes were documented, involved inhalation.

Materials and methods

Chemicals
[³H]-(±)-Nicotine ($61 \cdot 2$ Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA) and purity was determined by a thin-layer chromatography system (isobutanol–chloroform–ammonium hydroxide, 70:30:0·25). (+)-Nicotine and (−)-cotinine were generously donated by Dr E L. May and (+)-atropine was generously donated by Dr S. E. Robinson (both at Medical College of Virginia, Richmond, VA). (−)-Nicotine was purchased from ICN K & K Laboratories (Plainview, NY). The following drugs were purchased from Sigma Chemical Co. (St. Louis, MO): cytisine, (−)-lobeline, arecoline, oxotremorine, mecamylamine and (−)-atropine. Dyflos was obtained from Aldrich Chemical Company (Milwaukee, WI).

Animals and treatment
Dublin ICR male mice (Dominion Laboratories, Dublin, VA, 30–40 g, were exposed to the vapour of either dyflos or vehicle (propylene glycol) in an exposure apparatus and using the procedure described by Scimeca et al 1985). The vapour was generated by passing air at a flow of 750 mL min^{-1} over dyflos (4 mg) in propylene glycol (16 mg) which was heated to 80°C. The mice were allowed to breathe this

dyflos vapour by nose only for 5 min. Prior dosimetry experiments have shown that mice receive a dose of 2 mg dyflos kg^{-1} under these exposure conditions (Scimeca et al 1985). After the exposure the animals were removed from their holders and returned to their cages with free access to food and water. The animals remained in the laboratory until they were decapitated at 20 min, 10 or 24 h after exposure. Mice that were administered dyflos (2 mg kg^{-1}), or saline, by i.v. injection (tail vein) were decapitated 20 min after injection.

[^{3}H]Nicotine binding

[^{3}H]-($\pm$)-Nicotine binding was performed using the original method of Romano & Goldstein (1980), with some slight modifications (Costa & Murphy 1983). Whole mouse brain homogenate (minus cerebellum) was prepared in 10 volumes of ice-cold 0·05 M Na-K phosphate buffer (pH 7·4) and centrifuged (17 500 g, 4 °C) for 30 min. The pellet was suspended in 20 volumes of cold glass-distilled water, and then was allowed to remain on ice for 60 min before being centrifuged as described above. The pellet was then resuspended to a final concentration of 40 mg tissue mL^{-1} of buffer which resulted in a final protein content per tube of 507 $\pm$ 25 μg (mean $\pm$ s.e.m.). An aliquot (0·5 mL) of this membrane suspension was added to 0·5 mL buffer containing [^{3}H]nicotine at the appropriate concentration, in the absence or presence of 1 mM (−)-nicotine. Specific binding was defined as the difference in the binding of [^{3}H]nicotine in the absence or presence of 1 mM (−)-nicotine (each concentration was evaluated in triplicate). This concentration of (−)-nicotine was necessary for the complete saturation of the second binding component. Tubes were incubated at 37°C for 40 min, chilled on ice and rapidly filtered through Whatman GF/C filters (previously soaked in 0·1% solution of poly-L-lysine). Filters were washed three times with 4 mL ice-cold buffer. Filtration and washes were carried out in a cold room (4 °C) using manifolds and plates (Millipore Corp., Bedford, MA) that were pre-chilled. After drying, the filters were placed in 10 mL of scintillant, vigorously shaken for 1 h, and counted for radioactivity by liquid scintillation spectrometry. Data from these experiments were transformed by the method of Scatchard (1949) and analysed by the graphic method of Rosenthal (1967) to estimate the K$_D$ and B$_{max}$ values.

The ability of various compounds to inhibit [^{3}H]nicotine (5 and 115 nM) binding was determined by performing the binding assay in the presence of different concentrations of the test compounds. All compounds were made in glass-distilled water and 10 μL of the solutions were added to the incubation tubes.

Protein was measured using the Bradford (1976) method with bovine serum albumin as the standard.

Results

Characteristics of the nicotine binding sites

In a typical binding experiment with concentrations of [^{3}H]nicotine from 1 to 100 nM, specific binding ranged between 35 and 60% of the total binding, with the greater percentage of specific binding occurring at the lower concentrations of [^{3}H]nicotine. A plot of the specific binding versus

drug concentration indicated that complete saturation was reached at the highest concentration (Fig. 1A). Scatchard analysis of the binding data revealed a biphasic curve that could be resolved into two components (a representative Scatchard plot is presented in Fig. 1B). The average ($\pm$ s.e.m.) of three binding experiments with naive animals produced a high-affinity site with a B$_{max}$ of 11·8 $\pm$ 3·5 fmol (mg protein)$^{-1}$ and K$_D$ of 6·1 $\pm$ 2·5 nM, whereas the low-affinity site had a B$_{max}$ of 182·1 $\pm$ 23·5 fmol (mg protein)$^{-1}$ and K$_D$ of 114·2 $\pm$ 13·2 nM. The Hill coefficient was determined to be 0·85 thus indicating the existence of multiple, non-interacting binding sites or negative cooperativity.

The pharmacological characteristics of the [^{3}H]nicotine binding site were examined through the ability of various compounds to displace the binding of the radioligand (Table 1). Nicotinic agonists were determined to be much more potent than nicotinic antagonists in their ability to compete for [^{3}H]nicotine binding at a concentration of 5 nM. Cytisine, a ganglionic agonist, was four times more potent than (−)-nicotine. Other agonists, such as lobeline and (+)-nicotine, were also effective inhibitors. In contrast, the nicotinic antagonists mecamylamine and pempidine were at least
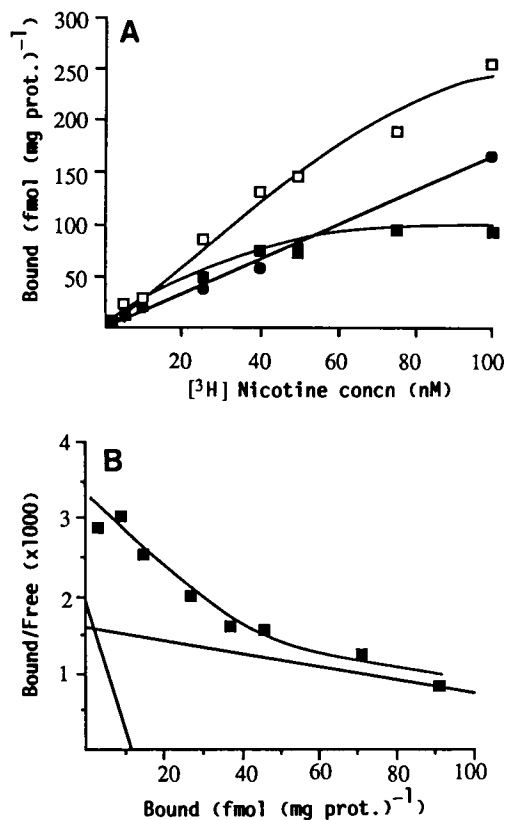

Fig. 1. Binding of [^{3}H]($\pm$)-nicotine to mouse brain homogenate. A. Saturation binding of [^{3}H] nicotine as a function of ligand concentration. Specific binding (■) is the difference between total binding (□) and non-specific binding (●). Non-specific binding was determined by the presence of 1 mM (−)-nicotine. The results shown are the mean of three experiments, with each concentration analyzed in triplicate. B. Scatchard analysis of [^{3}H]nicotine saturation binding. The data shown are from a typical binding experiment. Apparent K$_D$ and B$_{max}$ values for the high affinity (3·6 nM, 11·4 fmol are from a mg prot.)$^{-1}$ and low affinity (104 nM, 168 fmol (mg prot.)$^{-1}$) sites were determined through analysis of the data by the graphic method of Rosenthal.

Table 1. Inhibition of [³H]nicotine binding by various compounds.

| Compound | IC50 (μM)[a] | |
	5 nM [³H]Nicotine	115 nM [³H]Nicotine
Cytisine	0·006 ± 0·002 (4)	> 10 (2)
(−)-Nicotine	0·024 ± 0·003 (4)	> 10 (2)
(−)-Lobeline	0·054 ± 0·014 (5)	> 100 (2)
(+)-Nicotine	0·309 ± 0·034 (4)	> 10 (2)
Arecoline	57 ± 11 (2)	> 100 (1)
Oxotremorine	> 100 (2)	
(−)-Atropine	> 100 (2)	
(+)-Atropine	> 100 (2)	
Mecamylamine	> 100 (3)	> 100 (1)
Pempidine	> 100 (2)	> 100 (1)
(−)-Cotinine	> 100 (2)	
Dyflos	> 100 (3)	

[a] The IC50's were calculated by plotting the percent inhibition against the negative logarithm of the molar concentration. Typically six concentrations of drug, evaluated in triplicate, were used for each IC50 determination. The mean ± s.e.m. are presented with the number of experiments indicated in parentheses.

1500-fold less potent than nicotinic agonists, such as lobeline. Dyflos was found to be a poor displacer of [³H]nicotine binding, as was cotinine, the principal metabolite of nicotine. As expected, muscarinic agonists and antagonists were poor inhibitors. Stereospecificity of the binding was demonstrated by the finding that (+)-nicotine was thirteen times less potent in displacing [³H]nicotine than its stereoisomer, (−)-nicotine. All of the compounds tested were poor displacers of 115 nM [³H]nicotine, a concentration at which 90% of the ligand is bound to low affinity sites.

Effects of DFP on nicotine binding sites

The parameters of [³H]nicotine binding to mouse brain homogenate from dyflos- or vehicle-treated animals are presented in Table 2. It was previously shown that this treatment of dyflos produced a 75% inhibition of cholinesterase in the mouse brain within 5 min and which remained inhibited at this level for 12 h (Scimeca et al 1985). After acute dyflos treatment, a statistically significant alteration from vehicle-treated mice was produced in the low-affinity binding parameters at 24 h. The low-affinity receptor density was decreased by 60% while the affinity constant was decreased by 50%. However, no changes in the low-affinity binding parameters from controls at either 20 min or 10 h after treatment were observed. Additionally, no changes in

Table 2. Effect of dyflos on nicotine binding sites in mouse brain.

| Treatment[b] | Time | High-affinity | | Low-affinity | |
		K_D (nM)	B_{max} (fmol mg⁻¹)	K_D (nM)	B_{max} (fmol mg⁻¹)
Naive		6·1 ± 2·5[a]	11·8 ± 3·5	114 ± 13	182 ± 24
Vehicle		14·3 ± 3·5	13·4 ± 2·3	422 ± 84	316 ± 25
Dyflos	20 min	7·0 ± 2·2	8·8 ± 1·1	458 ± 85	379 ± 32
Vehicle	10 h	5·7 ± 3·1	6·1 ± 2·0	149 ± 23	132 ± 7
Dyflos	10 h	4·2 ± 0·5	4·5 ± 0·6	162 ± 40	160 ± 51
Vehicle	24 h	6·0 ± 1·6	8·3 ± 0·3	211 ± 26	259 ± 35
Dyflos	24 h	12·0 ± 3·8	10·8 ± 4·4	108 ± 26[c]	108 ± 25[c]
Saline, i.v.	20 min	6·0 ± 1·9	5·0 ± 0·4	122 ± 18	139 ± 14
Dyflos, i.v.	20 min	6·8 ± 0·8	6·9 ± 1·5	137 ± 12	129 ± 21

[a] The results are the means ± s.e.m. of three or four determinations. The K^D (nM) ad B_{max} (fmol(mg protein)⁻¹ values were derived from Scatchard analysis of the binding data.
[b] All treatments were administed by inhalation unless otherwise noted. The times that the animals were killed following treatment are indicated in the parentheses.
[c] Significantly different from vehicle treatment, P < 0·05 (Student's t-test).

the high-affinity binding parameters from controls were observed at any of the time points.

Alterations were also observed in the low-affinity binding parameters of the vehicle-treated animals as compared to naive mice at 20 min after the inhalation exposure procedure. It was possible that these changes might be related to the stressful exposure procedure, which might mask any possible drug-induced alterations at this time point. Therefore, animals were administered dyflos or saline by i.v. injection in what was felt to be a less stressful procedure. The binding parameters obtained after i.v. dyflos treatment were no different than those of the saline-treated animals. However, it should be noted that this administration procedure did not produce the alterations seen in the low-affinity binding parameters after the inhalation procedure.

Discussion

While the presence of nicotinic receptors in the CNS is generally well accepted, there is some dispute as to the number of sites and their characteristics. A number of studies have used radiolabelled nicotine to measure nicotinic sites in mammalian brain (Abood et al 1980; Romano & Goldstein 1980; Marks & Collins 1982; Costa & Murphy 1983; Sloan et al 1984) and in human brain obtained at autopsy (Shimohama et al 1985). Support for the cholinergic nature of [³H]nicotine binding has come from studies that have employed the radioligand [³H]acetylcholine (Schwartz & Kellar 1983, 1985). In addition, the binding properties measured by nicotine and acetylcholine are similar to each other when assessed by autoradiographic techniques (Clarke et al 1985). In the present study the specific [³H]nicotine binding to mouse brain homogenate was saturable, reversible, stereospecific, and inhibited by a number of nicotinic compounds. Scatchard analysis of the binding data produced a curvilinear plot that was resolved by the graphic method of Rosenthal (1967) into high- and low-affinity binding sites. Non-linear Scatchard plots have also been reported by other investigators (Yoshida & Imura 1979; Romano & Goldstein 1980; Costa & Murphy 1983; Sloan et al 1984; Shimohama et al 1985; Larsson & Nordberg 1985). In contrast, Marks & Collins (1982) found only one high-affinity binding site in the mouse brain, although they detected a second, low-affinity site in the presence of 1 mM unlabelled nicotine with the binding performed at 4 °C (instead of 10 μM unlabelled nicotine at 20 or 37° C). The samples in the present study were chilled on ice after incubation and then filtered and washed at 4 °C, as described in the original method of Romano & Goldstein (1980). Thus it appears that there was some effect of temperature that was responsible for the appearance of the low-affinity site.

Nicotinic agonists, such as cytisine, (−)-nicotine, and (+)-nicotine were potent inhibitors of [³H]nicotine (5 nM) binding. However, nicotinic antagonists, such as mecamylamine and pempidine, were very poor displacers of radioligand binding. This ineffectiveness of nicotinic antagonists has also been observed by several other investigators (Romano & Goldstein 1980; Abood et al 1980; Sershen et al 1981; Marks & Collins 1982; Costa & Murphy 1983). A number of theories have been proposed to explain this phenomenon, although concrete evidence supporting any

one explanation is still lacking. Stereospecificity was also observed in the inhibition of [^{3}H]nicotine binding, with ($-$)-nicotine thirteen-fold more potent than ($+$)-nicotine. Although some investigators (Romano & Goldstein 1980; Costa & Murphy 1983) found a good degree of stereospecificity in [^{3}H]nicotine binding, still others have found a low degree of stereospecificity (Sershen et al 1981; Martin & Aceto 1981). However, a lack of agreement regarding stereospecificity of nicotine binding has also been found in the pharmacological stereospecificity of nicotine (Martin & Aceto 1981).

A decrease in [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to muscarinic cholinergic receptor sites has been reported after chronic treatment with dyflos (Uchida et al 1979; Schiller 1979; Ehlert et al 1980; Sivam et al 1983; Yamada et al 1983; Lim et al 1986) and with another anticholinesterase, Tetram (Gazit et al 1979). For the most part these changes have been due to a decrease in muscarinic receptor density, without any changes seen in receptor affinity. Sivam et al (1983) have also investigated the effect of acute treatment with dyflos on [^{3}H]QNB binding to rat striatum. No changes were seen in any binding parameters measured at several time points except for a slight decrease in B_{max} at 24 h after the administration of 2 mg kg^{-1} dyflos.

These minimal effects of acute treatment with dyflos on central muscarinic receptors are similar to the effects on the nicotinic receptor presented here. Its acute administration caused only a slight effect on [^{3}H]nicotine binding to mouse brain homogenate at a dose that was previously shown to have profound effects on central cholineterase activity, as well as long-lasting effects on body temperature and motor coordination (Scimeca et al 1985). The decrease in receptor density at the low-affinity site is consistent with a theory of receptor down regulation in response to elevated levels of acetylcholine; however, the simultaneous increase in receptor affinity does not fit this theory. In addition, the possible functional significance of the low-affinity site is in doubt since competitive inhibition experiments with [^{3}H]nicotine at 115 nM failed to characterize the binding site as nicotinic. This is unlike the high-affinity site which has been characterized as nicotinic cholinergic in this study and in other studies (Romano & Goldstein 1980; Marks & Collins 1982; Costa & Murphy 1983) and which possesses similar binding parameters as [^{3}H]acetylcholine (Schwartz et al 1983; Marks & Collins 1982).

In summary, the results of this study show saturable and specific binding of [^{3}H]nicotine to mouse brain homogenate. Analysis of the binding data revealed the presence of high- and low-affinity sites, the former of which was characterized as nicotinic cholinergic. Furthermore, it appears that acute treatment with the anticholinesterase dyflos produced only a minimal effect on the low-affinity binding site, without any change in the high-affinity site. In the light of the small changes seen only in the low-affinity binding site after dyflos treatment, it appears unlikely that alterations in the density or affinity of nicotinic cholinergic receptors are responsible for the adaptation that occurs with cholinesterase inhibition in the mouse following acute DFP administration.

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