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Comparative Behavioral Effects of CNS Cholinesterase Inhibitors[1]

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MOSS, D. E., L. A. RODRIGUEZ AND S. B. McMASTER. *Comparative behavioral effects of CNS cholinesterase inhibitors*. PHARMACOL BIOCHEM BEHAV 22(3) 479–482, 1985.—Phenylmethanesulfonyl fluoride, methanesulfonyl fluoride, and physostigmine were compared on the efficacy with which each could suppress methylphenidate-induced stereotyped gnawing, an extrapyramidal motor behavior. Whereas physostigmine produced powerful suppression of the stereotypy, the sulfonyl fluorides did not produce any clear behavioral effect. Biochemical experiments conducted with the behavioral tests demonstrated that the sulfonyl fluorides produced inhibition of whole brain, caudate, cortex, cerebellum, hippocampus and brain stem cholinesterate equal to that produced by physostigmine. The reason for the marked discrepancy between the behavioral effect of physostigmine and the sulfonyl fluorides is unknown. It is, however, clear that the effect of the various drugs on extrapyramidal motor behaviors is not a simple function of the degree to which each inhibited CNS cholinesterase.

Cholinesterase Motor behavior Stereotypy Physostigmine Sulfonyl fluorides Methylphenidate Huntington's disease Tardive dyskinesia Extrapyramidal system Alzheimer's disease

CHOLINESTERASE inhibitors that act within the central nervous system (CNS) are of both theoretical and clinical interest. Research on physostigmine, for example, has shown that CNS active cholinesterase inhibitors may have potential clinical efficacy in such CNS cognitive disorders as Alzheimer's disease [2,3], or extrapyramidal motor disorders such as Huntington's disease [7,10], and tardive dyskinesia [5,8].

The actual clinical use of cholinesterase inhibitors has, however, been limited because they are not generally selective for cholinesterase within the CNS. Therefore, cholinesterase inhibition in the peripheral nervous system (e.g., autonomic ganglia, postganglionic fibers of the parasympathetic system, somatic myoneural junctions) can produce severely toxic side effects. In order to avoid these peripheral effects, there has been renewed interest in the identification or development of compounds that are relatively selective for cholinesterase within the CNS [3].

Recent research has demonstrated that certain sulfonyl flourides, irreversible cholinesterase inhibitors similar to organic phosphates, are relatively selective for CNS enzyme. Although the biochemical mechanism for the selectivity of the sulfonyl fluorides is unknown, under certain circumstances, they can produce up to 90% inhibition of CNS cholinesterase with a maximum of 30% inhibition of peripheral enzyme [16]. These compounds can produce a pharmacologically significant effect in the CNS without significant peripheral toxicity [16].

The purpose of the present research was to compare the efficacy of two selected sulfonyl fluorides with that of physostigmine on psychostimulant-induced stereotypy. This specific behavior was selected because it is a relatively well understood motor behavior controlled by the extrapyramidal system (basal ganglia) [1,9]. Secondly, the extrapyramidal system is thought to be involved in Huntington's disease and tardive dyskinesia, two disorders in which a CNS selective cholinesterase inhibitor, similar to those being studied, might have potential clinical utility [17]. Lastly, this behavior is exquisitely sensitive to physostigmine [4,13] and, therefore, it can provide a standard against which to compare the behavioral effects of other cholinesterase inhibitors. Contrary to the expected results, however, the sulfonyl fluorides were discovered to be without any significant effect in this behavioral model. The origin of this paradoxical result is unknown. However, it suggests that some behavioral effects may be caused by a pharmacological action unrelated to cholinesterase inhibition.

EXPERIMENT I—COMPARISON OF PHYSOSTIGMINE AND SULFONYL FLUORIDES ON STEREOTYPED GNAWING

The first objective of Experiment I was to compare the suppression of methylphenidate-induced gnawing produced by physostigmine with that of the sulfonyl fluorides. The second objective was to relate cholinesterase-induced suppression of gnawing to specific levels of cholinesterase inhibition produced in the CNS.

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METHOD

The subjects were 49 female Sprague-Dawley albino rats reared and maintained with ad lib food and water in a 12 hour light/dark cycle (on at 1800) in the animal colony at the University of Texas at El Paso (UTEP). Female rats were used because they show more reliable methylphenidate-induced stereotyped gnawing at lower doses (30–40 mg/kg) than do males (60 mg/kg or more) [14]. The animals were reared in the UTEP animal colony in order to insure that they were never exposed to cholinesterase inhibiting pesticides (e.g., parathion, malathion, etc.) because such exposure would have invalidated the results.

Stereotyped gnawing was recorded according to the automated UTEP procedure described in detail earlier [14]. Briefly, this procedure is based on the observation that stereotyped gnawing includes grasping the object and pulling up vigorously. Gnawing can, therefore, be easily and accurately measured by connecting a microswitch to a piece of hardware cloth (wire mesh 12.5 cm square) anchored loosely to the floor of the gnawing chamber (23 cm square) and counting the number of times the microswitch is operated [14].

Stereotyped gnawing was induced by IP injection of 40 mg/kg methylphenidate HCl (a generous gift from CIBA-GEIGY, Summit, NJ). This dose was sufficient to cause gnawing in approximately 80% of the control animals at a mean rate of 1352 counts (SEM 172) during the first hour following the injection. Because of behavioral variability, controls (methylphenidate alone) were tested with all groups and the results were expressed as a percentage of the concurrent control animals.

Physostigmine salicylate (PHYSO; Sigma Chemical, St. Louis) was prepared in deionized water and studied in detail at several doses up to 1.0 mg/kg. Because carbamylating inhibitors such as PHYSO produce inhibition quickly and are very short acting, PHYSO was injected 20 min prior to behavioral testing. Phenylmethanesulfonyl fluoride (PMSF; also known as phenylmethylsulfonyl fluoride and alpha-toluenesulfonyl fluoride; Calbiochem, San Diego) and methanesulfonyl fluoride (MSF; Aldrich, Milwaukee) were selected as representative sulfonyl fluorides. PMSF (85 mg/kg) and MSF (1.5 mg/kg) were dissolved in sasame oil and, because the sulfonyl fluorides are irreversible long-lasting inhibitors, these compounds were injected 18 hr prior to testing. These specific doses of sulfonyl fluorides were selected for detailed study because pilot data demonstrated that lower doses did not affect gnawing and these high doses produced up 60–70% inhibition of CNS cholinesterase, a level that should have produced approximately 90% suppression of gnawing. The specific times of injection and testing for all drugs were selected to coincide with maximum CNS cholinesterase inhibition. All drugs were prepared daily and injected IP in a volume of 1 ml/kg or less.

In order to correlate gnawing behavior with CNS enzyme inhibition, all animals were decapitated immediately after the one hour gnawing test and assayed for cholinesterase inhibition according to the spectrophotometric method of Ellman *et al.* [6] as described in detail earlier [15]. Briefly, the brains were quickly removed and placed in ice cold 0.1 M (Na) PO_4 buffer, homogenized as a 20% w/v suspension, diluted 1:10 in additional buffer, and assayed in triplicate for enzyme activity at 500 μM acetylthiocholine substrate at pH 7.0, 25°. Because the K_m for rat brain cholinesterase under these assay conditions is 50–55 μM and the enzyme follows classical Michaelis kinetics, assays conducted at 500 μM produced about 90% of V_{max}, an excellent estimate of the amount of enzyme activity present in the brain samples. Because the carbamylated enzyme produced by PHYSO has a half-life of only about 30–60 min at 25–37° [11], it was critical to keep the enzyme sample cold and complete the assays as quickly as possible. All assays were completed within 1.5 hr of the time of death and no significant reactivation of enzyme inhibited by PHYSO was observed in ice cold samples during that period of time. Although the sulfonylated enzyme produced by the sulfonyl fluorides does not undergo any significant spontaneous reactivation like that observed with PHYSO, all brain samples were processed according to the same procedures. The level of enzyme activity observed in the assays appeared to be good estimates of actual *in vivo* conditions during the gnawing tests.

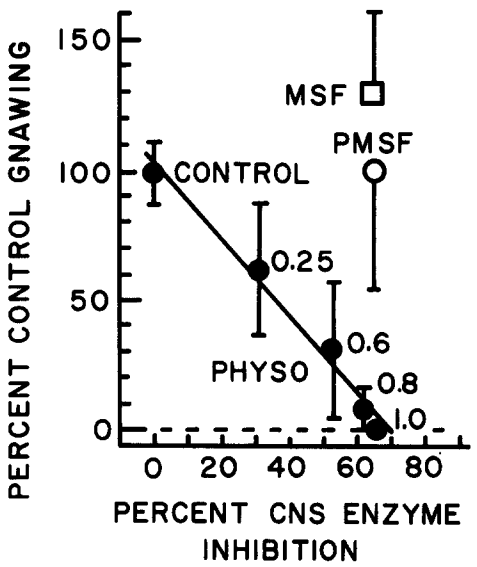

FIG. 1. The effect of physostigmine, phenylmethanesulfonyl fluoride, and methanesulfonyl fluoride on methylphenidate-induced gnawing. The doses of physostigmine (PHYSO in mg/kg) are shown in the figure. The mean level of methylphenidate-induced gnawing (40 mg/kg) is plotted against the level of CNS cholinesterase inhibition produced by each dose of PHYSO (filled circles), 85 mg/kg PMSF (open circle), or 1.5 mg/kg MSF (open square). The error bars represent one SEM. The correlation between the mean level of gnawing and the mean cholinesterase inhibition produced by PHYSO was −0.9768.

RESULTS

As shown in Fig. 1, PHYSO in various doses up to 1.0 mg/kg have a strong suppressive effect on methylphenidate-induced gnawing. The correlation between the mean level of gnawing and the mean level of CNS cholinesterase inhibition was −0.9768, $p<0.01$. Surprisingly, doses of the sulfonyl fluorides that produced 64.44% (PMSF) and 64.12% (MSF) CNS cholinesterase inhibition did not produce any signifi-

TABLE 1
EFFECT OF VARIOUS INHIBITORS OF LOCAL BRAIN AREAS

	(Data Expressed as Percent of Control Enzyme Activity)*		
Brain Area	1 mg/kg PHYSO	85 mg/kg PMSF	1.5 mg/kg MSF
Caudate	20.2% (1.8%)	31.0% (8.0%)	22.8% (3.8%)
Hippocampus	25.6% (1.3%)	32.4% (4.5%)	27.1% (5.6%)
Cortex	31.3% (1.4%)	36.2% (4.1%)	30.4% (4.3%)
Cerebellum	30.0% (5.8%)	38.5% (1.2%)	32.4% (2.5%)
Brain Stem	27.3% (2.2%)	32.8% (4.0%)	26.6% (3.5%)

*N=4 in all groups. SEM shown in parentheses.

cant suppression of gnawing (Fig. 1). Although the level of gnawing predicted from linear regression analysis of the PHYSO data was only 11% of control, PMSF treated animals showed 100.9% and MSF treated animals showed 130.1% of control (methylphenidate alone) gnawing. Analysis of variance [18] indicated that there was no difference between the gnawing rates observed in the control, PMSF, and MSF treated animals, $F(2,12)=0.159$, n.s.

EXPERIMENT II—COMPARISON OF THE EFFECTS OF PHYSO, PMSF, AND MSF ON ENZYME ACTIVITY IN SELECTED BRAIN AREAS

Because the results of Experiment I demonstrated a dramatic difference in the effects of PHYSO as compared to the sulfonyl fluorides on gnawing behavior in spite of the finding of equivalent inhibition of whole brain cholinesterase activity, Experiment II was conducted to determine if these various compounds might be producing different levels of inhibition in local brain areas.

METHOD

An additional 20 animals, matched in sex and type to those used in Experiment I, were treated with 1.0 mg/kg PHYSO (N=4), 85 mg/kg PMSF (N=4), 1.5 mg/kg MSF (N=4), or vehicle alone (N=4) according to the procedures used in Experiment I. The brains of these animals were dissected to remove samples of caudate/putamen, cerebellum, brain stem, hippocampus, and cortex for individual assays of inhibition in these local brain areas. The enzyme assays were conducted in accordance with the procedures described above except that, because of the small size of the samples, the tissues were homogenized directly as a 4% w/v suspension (except caudate/putamen) and assayed without the additional 1:10 dilution. Because the caudate/putamen is the richest source of cholinesterase in the brain, these samples were homogenized as 0.67% (1:150) w/v suspension. These dilutions produce a convenient level of enzyme activity during the 2 to 4 minute assays.

RESULTS

The results obtained in Experiment II are shown in Table 1. Multiple analysis of variance [18] showed that there was not a significant difference in the overall amount of enzyme inhibition produced by 1.0 mg/kg PHYSO, 85.0 mg/kg PMSF, and 1.5 mg/kg MSF, $F(2,9)=1.615$, n.s. However, the different brain areas were inhibited to a significantly different degree, $F(4,36)=4.665$, $p<0.01$, but there was no interaction between brain area and inhibitors, $F(8,36)=0.190$, n.s.

GENERAL DISCUSSION

Experiment I replicated earlier results by others [4,13] showing the exquisite sensitivity of psychostimulant-induced behaviors to suppression by physostigmine. In spite of the sensitivity of the behavioral model, the results obtained in these experiments demonstrated a striking difference in the behavioral effects of various CNS cholinesterase inhibitors. These results are particularly interesting in view of the finding that the inhibition of CNS enzyme was virtually identical during the behavioral tests conducted with each compound.

Although the reason for these contradictory results are unknown, the most likely explanation appears to be that one class of inhibitor is having some additional pharmacological effect that is not shared by the other. There are several possibilities that will require additional research. These are that the different inhibitors are : (1) affecting different types of cholinesterases, (2) having additional, noncholinesterase-related effects on cholinergic function, and/or (3) affecting other neurotransmitter system(s).

With regard to the possibility that the two classes of inhibitors are affecting different forms of cholinesterase, it has been well established that the nervous system of vertebrates contain at least two general classes of cholinesterase. The major type is acetylcholinesterase (EC 3.1.1.7) while the alternate form is butyrylcholinesterase (EC 3.1.1.8). It is also clear that each general class of enzyme exists in a number of molecular forms that may correspond to distinctly different populations at the cellular level and, furthermore, the various molecular forms may have specific physiological functions [12]. One limitation of the present research is that the measurement of overall enzyme activity did not determine if one form of the enzyme is being inhibited more or less than other forms. Examination of this possibility should be a topic for further research as it would be quite important to determine if different types or molecular forms of the enzyme control different physiological functions to the extent that such contradictory results could be obtained.

The second possibility is that the two classes of enzyme inhibitor have some effect on cholinergic neurotransmission unrelated to direct effects on cholinesterase inhibition. There are several possibilities that merit consideration. Specifically, it is possible that cholinesterase inhibitors also interact with acetylcholine receptors. Certain substances could act as nicotinic or muscarinic agonists or antagonists while others would not. It is interesting that McMaster [13] demonstrated that methylphenidate-induced gnawing was strongly suppressed by a muscarinic agonist (oxotremorine) but enhanced by a nicotinic agonist (nicotine). In addition, it is possible that the sulfonyl fluorides affect cholinergic

enzymes other than cholinesterases. For example, choline acetyltransferase might also be inhibited and, therefore, the availability of acetylcholine might actually be reduced. Such effects could account for the contradictory results obtained and must not be ignored in future research.

Lastly, because of the powerful cholinergic-monoaminergic interactions in the extrapyramidal system, particularly in the control of motor behaviors [17], it is also impossible to rule out interactions between some cholinesterase inhibitors and other neurotransmitters. Likely candidates would include interactions with enzymes, transport mechanisms, or receptors related to dopamine, gamma-aminobutyric acid, or other neurotransmitter function.

The results presented in Experiments I and II demonstrate the potential clinical efficacy of CNS cholinesterase inhibitors may depend critically upon other pharmacological properties of the specific compounds being tested. Because virtually everything known about the behavioral effects of CNS cholinesterase inhibition has resulted from research conducted with physostigmine, the inability to replicate the results obtained with physostigmine by using other CNS cholinesterase inhibitors may introduce significant concerns about the interpretation of earlier research.

At the present time, PMSF and MSF do not appear to show the predicted promise for clinical applications in extrapyramidal motor disorders wherein physostigmine has produced some clinical effects [5, 7, 8, 10]. The inability to demonstrate potential efficacy for the sulfonyl fluorides in motor behaviors may not, however, limit the potential utility of these compounds in enhancement of cognitive functioning in such disorders as senile dementia of the Alzheimer type [2,3]. In fact, the absence of powerful effects on the extrapyramidal motor system may enhance the value of certain sulfonyl fluorides in cognitive applications.

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