21. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
22. E. M. Brown, S. A. Fedak, C. J. Woodard, G. D. Aurbach and D. Rodboard, *J. biol. Chem.* **251**, 1239 (1976).
23. R. S. Salter, M. M. Krinks, C. B. Klee and E. J. Neer, *J. biol. Chem.* **256**, 9830 (1981).
24. R. J. Lee, D. B. Evans, S. M. Baky and R. J. Laffan, *Eur. J. Pharmac.* **33**, 371 (1975).
25. E. Miyamoto, J. F. Kuo and P. Greengard, *J. biol. Chem.* **244**, 6395 (1969).
26. C. S. Rabe, J. Schneider and R. McGee, *J. Cyclic Nucleotide Res.* **8**, 371 (1982).

Biochemical Pharmacology, Vol. 33, No. 11, pp. 1823–1825, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Effects of diisopropylfluorophosphate (DFP) and other cholinergic agents on release of endogenous dopamine from rat brain striatum *in vitro**†

(*Received* 6 *July* 1983; *accepted* 28 *November* 1983)

Diisopropylfluorophosphate (DFP) is a neurotoxic organophosphate whose major mechanism of action is the irreversible inhibition of acetylcholinesterase, the enzyme that catabolizes acetylcholine. The subsequent buildup of acetylcholine at central and peripheral synapses is thought to be the primary cause of the physiological and behavioral perturbations observed following DFP administration [1]. While cholinergic synapses may be the major site of action of DFP, there is reason to believe that other neurochemical systems might be involved in the acute and/or delayed effects of organophosphate neurotoxins including DFP [2, 3]. Recovery of acetylcholinesterase activity does not always parallel return to normal function [3, 4].

DFP could directly affect neuronal systems by inhibition of serine active-site enzymes other than acetylcholine. Peptidases that possibly regulate peptidergic neurons are a likely target [5]. Also, other metabolically important enzymes or membrane proteins that function as receptors, ion channel regulators, or uptake sites might be directly attacked [2, 6]. DFP could also indirectly involve other neurotransmitter systems via its action on acetylcholine. Cholinergic drugs have been shown to affect norepinephrine turnover and release in the cortex and hypothalamus [7–11]. Cholinergic–dopaminergic interactions in the striatum have been described [12–14]. Organophosphates have been reported to alter catecholamine levels and turnover in rat brain [15].

As part of our investigations into the neurochemical mechanisms of action of organophosphates, we decided to examine the effects of DFP on the release of endogenous striatal dopamine using a sensitive technique we utilized previously to measure the small amounts of endogenous catecholamines released from rat brain regions *in vitro* [16–19].

Materials and methods

Animals. Male Sprague–Dawley rats (300–400 g) were used. Animals were individually housed in a temperature- and light-controlled room with food and water freely available.

Drugs. DFP and nicotine bitartrate were obtained from ICN Pharmaceuticals, Plainview, NY. Mecamylamine hydrochlochloride was supplied by Merck, Sharp & Dohme, West Point, PA. Physostigmine sulfate, oxotremorine sesquifumarate, and atropine sulfate were purchased from the Sigma Chemical Co. St. Louis, MO. DFP was first diluted to 5×10^{-2} M in 95% saline/5% ethanol. Other drugs were first prepared as 5×10^{-2} M solutions in saline. Further dilutions were made for the experiments that required final drug concentrations of less than 10^{-3} M drug. Twenty microliters of drug dilution was then added to 1.0 ml of incubation buffer.

Experimental procedures. Rats were decapitated and the corpora striata were dissected on ice. Tissues from six rats were combined, weighed and chopped using a McIlwain tissue chopper (0.3 mm × 0.3 mm). The pooled tissue was washed twice in 25 ml of cold Krebs buffer (120 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose, 10^{-5} M nialamide, 0.1 mg/ml ascorbic acid, pH 7.4, gassed with 95% O_2/5%CO_2) and then resuspended in 26 ml of fresh cold buffer. Aliquots (1 ml) of tissue suspension were pipetted into each of twenty-four 14-ml polypropylene test tubes. The tubes were centrifuged, and the supernatant fractions were discarded, yielding twenty-four similar tissue pellets. These were incubated with 1.0 ml of 37° buffer.

In the first experiment, one drug (DFP, physostigmine, atropine, oxotremorine, nicotine, mecamylamine) was tested in each twenty-four tube incubation with six tubes receiving no drug, six tubes receiving 10^{-5} M drug, six tubes receiving 10^{-4} M drug, and six tubes receiving 10^{-3} drug. Tubes were incubated for 15 min following drug addition. Additional separate incubations were performed for each drug at each concentration with added KCl (45 mM), with the KCl added at the 5-min point in the incubation. Thus, a total of twelve incubations of twenty-four tubes each was performed. Tissue in each incubation was derived from a single pool of striatal tissue obtained from six rats. Following the 15-min incubation, release was terminated by centrifugation at 4°. Supernatant fractions were stored at −70° until assayed for dopamine.

In a second experiment performed to determine whether the effects of high DFP concentrations observed in the first experiment could be blocked by atropine, atropine (10^{-3} M) was added to tissue in 1 ml of warm buffer prepared as in experiment one above. Five minutes later, DFP (10^{-3} M) was added. Following an additional 5-min incubation at 37°, KCl was added, and the tubes were incubated for an additional 10 min. The tubes were then centrifuged as above.

Dopamine assay. The procedure of Coyle and Henry [20] was followed with some modifications. The supernatant aliquot (0.1 ml) was acidified with 20 μl of 0.5 N perchloric acid. Additional magnesium and ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) were added to the reaction mixture to counteract the inhibitory effects of the calcium in the buffer on the activity of catechol-*O*-methyl transferase. All drugs used in the experiments were tested in the assay to determine possible interference. Only physo-

* In conducting the research described in this report, the investigators adhere to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

† The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense (Para. 4–3, AR 360–5).

stigmine affected the assay. Physostigmine 'blanks' were used to calculate data when physostigmine-treated samples were assayed.

Statistics. Comparisons between groups were made using Student's *t*-test.

Results

Since the various drugs were tested in separate incubations derived from separate tissue pools, all comparisons were made between drug and co-incubated controls. Spontaneous release of striatal DA was not affected significantly by 10^{-4} M and 10^{-5} M concentrations of all drugs tested with the exception of 10^{-4} M atropine which significantly decreased DA release as shown in Table 1. Although DFP appeared to increase spontaneous DA release at 10^{-4} M and 10^{-5} M concentrations, these increases were not statistically significant. At a 10^{-3} M concentration, DFP inhibited spontaneous DA release to 31% of control while mecamylamine and especially nicotine increased spontaneous DA release.

Potassium increased release of endogenous DA by approximately 370% as compared to controls incubated without added KCl. Potassium-stimulated release of striatal DA was affected significantly by DFP, physostigmine, atropine, oxotremorine and nicotine (Table 2). DFP markedly decreased potassium-stimulated striatal DA release with release decreased to 76% at 10^{-5} M DFP, 54% at 10^{-4} M DFP and to 12% of control by 10^{-3} M DFP. Physostigmine (10^{-4} M) also reduced potassium-stimulated DA release. Atropine slightly increased release at 10^{-4} M and decreased release at 10^{-3} M. Oxotremorine (10^{-3} M) decreased release while nicotine (10^{-3} M) slightly increased release.

Addition of atropine (10^{-3}) prior to addition of DFP (10^{-3} M) only slightly blocked the DFP inhibition of potassium-stimulated DA release: control, 648 ± 47 ng DA released/g tissue; DFP, 56 ± 10 ng DA released/g tissue; atropine plus DFP, 134 ± 17 ng DA released/g tissue.

Discussion

We were able to measure the relatively small absolute amounts of dopamine released from striatum *in vitro* by using a sensitive assay procedure. This method avoids several problems associated with the more commonly used technique of estimating endogenous release by measuring the release of exogenous radiolabeled catecholamine previously incubated with and taken up by the tissue of interest.

Increasing potassium concentration causes depolarization of neuronal tissue and acts as a stimulus for release of neurotransmitters. In our experiments, 45 mM KCl consistently elevated endogenous DA release.

DFP markedly inhibited the release of endogenous DA. Both spontaneous release and potassium-stimulated release were reduced by 10^{-3} M DFP, but the effect on potassium-stimulated release was seen at lower DFP concentrations and was dose related.

Since DFP increases synaptic availability of acetylcholine, we examined the ability of both a cholinergic

Table 1. Effects of DFP and other cholinergic agents on spontaneous release of DA from striatum *in vitro**

Agent	DA released (% control)			
	Control†	10^{-5} M	10^{-4} M	10^{-3} M
DFP	100 ± 30	133 ± 34	170 ± 36	31 ± 7‡
Physostigmine	100 ± 10	135 ± 35	119 ± 60	131 ± 59
Atropine	100 ± 14	87 ± 12	49 ± 13‡	136 ± 26
Mecamylamine	100 ± 7	106 ± 11	104 ± 9	157 ± 16‡
Oxotremorine	100 ± 19	132 ± 18	98 ± 31	132 ± 16
Nicotine	100 ± 28	91 ± 14	175 ± 39	289 ± 34‡

* Values represent the mean ± S.E.M. of six samples. KCl concentration = 5 mM for all tubes.

† Separate control tubes were incubated for each drug since each incubation set of twenty-four tubes was derived from a separate tissue pool. Average DA released in controls was 380 ng DA released/g striatal wet weight. The twenty-four samples representing control plus three concentrations of drug were incubated and assayed together. Comparisons are made within incubations.

‡ Differs from appropriate control, $P < 0.05$, Student's *t*-test, two-tailed.

Table 2. Effects of DFP and other cholinergic agents on potassium-stimulated release of DA from striatum *in vitro**

Agent	DA released (% control)			
	Control†	10^{-5} M	10^{-4} M	10^{-3} M
DFP	100 ± 13	76 ± 10	54 ± 4‡	12 ± 2‡
Physostigmine	100 ± 8	93 ± 18	39 ± 12‡	57 ± 26
Atropine	100 ± 6	111 ± 7	116 ± 4‡	78 ± 9‡
Mecamylamine	100 ± 14	95 ± 26	109 ± 14	118 ± 26
Oxotremorine	100 ± 21	68 ± 13	73 ± 15	56 ± 8‡
Nicotine	100 ± 11	120 ± 16	109 ± 12	127 ± 8‡

* Values represent the mean ± S.E.M. of six samples. KCl concentration = 45 mM.

† Separate control tubes were incubated for each drug since each incubation set of twenty-four tubes was derived from a separate tissue pool. Average control DA release was 1433 ng DA released/g striatal wet weight. The twenty-four samples representing control plus three concentrations of drug were incubated and assayed together. Comparisons are made within incubations.

‡ Differs from appropriate control $P < 0.05$, Student's *t*-test, two-tailed.

muscarinic agonist, oxotremorine, and a cholinergic nicotinic agonist, nicotine, to decrease endogenous DA release. We also examined the effects of two cholinergic antagonists, mecamylamine and atropine, and the effect of physostigmine, a carbamate-type cholinesterase inhibitor. Nicotine increased DA release, consistent with reports by others who measured the effect of nicotine on release of radiolabeled DA [21]. Therefore, DFP inhibition of DA release is probably not mediated via stimulation of nicotinic receptors by excess acetylcholine.

At 10^{-5}M and 10^{-4}M concentrations, the inhibitory effects of physostigmine and oxotremorine on potassium-stimulated DA release were similar in magnitude to those of DFP. Also, the apparent stimulatory effects of DFP at 10^{-5}M and 10^{-4}M on spontaneous DA release were generally similar to those of physostigmine and oxotremorine. However, the marked inhibitory effects of 10^{-3}M DFP on both spontaneous and potassium-stimulated DA release were not seen following either physostigmine or oxotremorine. In addition, the effects of high DFP concentrations were only slightly blocked by preincubation with atropine.

Taken as a whole, these data suggest that, at the moderate DFP concentrations, DFP effects on endogenous DA release may be attributable to the effects of increased acetylcholine on muscarinic sites located on DA neurons. However, at high DFP concentrations, DFP appeared to inhibit DA release to a greater extent than can be explained by the presence of excess acetylcholine.

Therefore, we suggest that at high concentrations DFP might directly attack protein sites that are important in the regulation of release of dopamine and possibly other neurotransmitters as well. In light of the diverse functions and wide distribution of catecholamine pathways, these effects might be of considerable importance in the mechanism of action of both acute and/or delayed neurotoxicity of DFP and possibly other organophosphates.

Acknowledgements—We wish to thank Golden C. Driver, SP5 Leigh Landman Roberts, and SP4 Terry Eggleston for expert technical support and Pat Conners for secretarial assistance.

* Requests for reprints should be addressed to: G. Jean Kant, Ph.D., Department of Medical Neurosciences, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307.

Department of Medical Neurosciences
Walter Reed Army Institute of Research
Washington, DC 20307, U.S.A.

G. JEAN KANT*
CLYDE C. KENION
JAMES L. MEYERHOFF

REFERENCES

1. G. B. Koelle, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. Goodman and A. Gilman), 5th Edn, pp. 445–66. Macmillan, New York (1975).
2. R. Jovic, H. S. Bachelard, A. G. Clark and P. C. Nicholas, *Biochem. Pharmac.* **20**, 519 (1971).
3. M. D. Kozar, D. H. Overstreet, T. C. Chippendale and R. W. Russell, *Neuropharmacology* **15**, 291 (1976).
4. R. C. Jovic, *Eur. J. Pharmac.* **25**, 159 (1974).
5. J. J. O'Neill, *Fund. app. Toxic.* **1**, 154 (1981).
6. K. Kuba and E. X. Albuquerque, *Science* **181**, 853 (1973).
7. G. H. Hall and D. M. Turner, *Biochem. Pharmac.* **21**, 1829 (1972).
8. F. R. Goodman, *Neuropharmacology* **13**, 1025 (1974).
9. T. A. Reader, J. de Champlain and H. Jasper, *Brain Res.* **111**, 95 (1976).
10. W. W. Morgan and K. A. Pfeil, *Life Sci.* **24**, 417 (1979).
11. K. Yoshida, Y. Kato and H. Imura, *Brain Res.* **182**, 361 (1980).
12. L. L. Butcher, *Life Sci.* **21**, 1207 (1977).
13. M. F. Giorgueiff, M. L. Le Floc'h, J. Glowinski and M. J. Besson, *J. Pharmac. exp. Ther.* **200**, 535 (1977).
14. M. F. Giorgueiff, M. L. Le Floc'h, T. C. Westfall, J. Glowinski and M. J. Besson, *Brain Res.* **106**, 117 (1976).
15. R. R. Fiscus and W. Van Meeter, *Fedn Proc.* **36**, 3589A (1977).
16. G. J. Kant and J. L. Meyerhoff, *Life Sci.* **23**, 2111 (1978).
17. G. J. Kant and J. L. Meyerhoff, *Life Sci.* **20**, 149 (1977).
18. J. L. Meyerhoff and G. J. Kant, *Life Sci.* **23**, 1481 (1978).
19. G. J. Kant, J. L. Meyerhoff and M. E. Corcoran, *Expl. Neurol.* **70**, 701 (1980).
20. J. T. Coyle and D. Henry, *J. Neurochem.* **21**, 61 (1973).
21. T. C. Westfall, *Neuropharmacology* **13**, 693 (1974).