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Failure of β-adrenergic receptors to modulate adrenal medullary secretion*

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The isolated bovine adrenal medullary (BAM) cell has proved to be a useful preparation for the study of neurosecretory processes [1–3]. BAM cells secrete several different biologically active compounds including the catecholamines (CAs) [dopamine, norepinephrine (NE), epinephrine (EPI)], purines (ATP, ADP), and opioid peptides [4]. It has been argued that one or more of these secreted products exert feedback effects on BAM cell secretion [5–8] in much the same way that NE released from adrenergic neurons can modulate its own release through presynaptic receptors [9]. All of these compounds share the potential to influence the level of adenosine-3′,5′-monophosphate (cAMP) which appears to be involved in modulating stimulus-secretion-coupling in several different systems [10, 11] including perfused adrenal glands [12, 13]. Zinder and Greenberg [14, 15] recently reported that BAM cell secretion could be modulated through β-adrenergic receptors. The β-adrenergic agonist isoproterenol (INE) reportedly stimulated CA secretion and potentiated acetylcholine (ACh)-stimulated secretion as did the addition of dibutyryl-cAMP. The β-adrenergic antagonist propranolol inhibited ACh-induced secretion. We have attempted to confirm these observations and have been unable to find evidence of either β-adrenergic modulation of secretion or intracellular cAMP accumulation. Our evidence suggests that the effects of propranolol on BAM cell secretion are due to membrane stabilization and not to β-adrenergic receptor blockade. In our experiments, INE failed to modulate either secretion or cAMP metabolism. We have confirmed that the addition of exogenous cAMP potentiates BAM cell secretion.

Methods

Cell culture. Bovine adrenals were obtained from a local slaughterhouse and transported on ice to the lab. BAM cell cultures were prepared essentially as described by Wilson and Viveros [16] except that the initial perfusion of the glands was omitted. The cells were plated on 24 × 16 mm Falcon multiwells at a density of 10^6 cells/well. The culture plates were incubated at 37° in a humidified atmosphere containing 5% CO_2. After 2 days in culture the medium was replaced with fresh, serum-free BAM cell medium. This medium was changed every 2–3 days.

Secretion experiments. BAM cells were used for secretion experiments between 3 and 7 days in culture. Culture plates were removed from the incubator and allowed to equilibrate with room air and temperature (25°). Since these cells attach themselves to the culture plate, the plates could be inverted to remove the medium. The plates with attached cells and free of culture media were quickly placed upside down on top of separate plates containing buffer B [150 mM NaCl, 4.2 mM KCl, 1.0 mM NaH_2PO_4, 11.2 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid (Hepes) (pH 7.4), 0.7 mM $MgCl_2$]. The plates were then inverted at t = 0. The secretion buffer was removed at the end of the secretion period by inverting the plates leaving the cells attached to the upper plate and the secretion buffer in the wells of the second plate. For all secretion experiments the cells were subjected to a 10-min preincubation in 1 ml of buffer B with or without addition of drugs. The preincubation was followed by a 10-min incubation in 1 ml of fresh buffer B containing various concentrations of carbamylcholine (CCh) and whatever drug was present during the preincubation. The incubation buffer was removed at the end of the 10 min and replaced with 0.1 N HCl to extract cellular CAs and cAMP. The incubation buffer was assayed separately for secreted CAs and cAMP. Protein was dissolved with 0.05 N NaOH and retained for subsequent analysis.

Catecholamine determination. Unless stated otherwise, CAs from both the secretion buffer and the acid extract were measured by the automated trihydroxyindole method described by Robinson and Watts [17] as modified by Ackerly *et al.* [18]. CAs from experiments using INE were quantified by high performance liquid chromatography with an electrochemical detector (HPLC/EC) as described by Miller *et al.* [19]. Frozen CA samples were thawed in the presence of alumina, alkali and 8 pmoles of internal standard (3,4-dihydroxybenzylamine). The samples were separated on an ODS 5 μm reverse phase C_{18} column and quantified by a Bioanalytical LC-4A electrochemical detector with a TL-5/glassy carbon electrode at 700 mV.

cAMP radioimmunoassay. Cellular cAMP content was measured by acetylating 0.5 ml of the 0.1 N HCl BAM cell extract. cAMP content was determined by the automated radioimmunoassay described by Brooker *et al.* [20]. cAMP is reported as pmoles/mg protein or pmoles/10^6 cells. Control and CCh-stimulated cAMP levels in both preincubation and incubation buffers were below the level of detection.

Protein determinations. Proteins were determined using the BioRad microassay as described by Bradford [21].

Statistical methods. Statistical differences between means were evaluated using Student's *t*-test. Significance was assigned to P values less than 0.05. "N" represents the number of culture wells for each experimental condition.

Materials

Carbamylcholine chloride, (−)-arterenol bitartrate (NE), (±)isoproterenol, collagenase type I, sodium penicillin-G, streptomycin and deoxyribonuclease I were purchased from the Sigma Chemical Co., Saint Louis, MO; 8-Bromo-adenosine-3′,5′-monophosphoric acid and adenosine-2′,3′-monophosphoric acid were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN.; epinephrine bitartrate was purchased from ICN Nutritional Biochemicals, Cleveland, OH; *l*-propranolol and *d*-propranolol were obtained from Ayerst Laboratories Inc., New York, NY; Dulbecco's minimum essential medium (DMEM), HAMs-F-12 and fetal bovine serum were purchased from the Grand Island Biological Co., Grand Island, NY; 5-fluorodeoxyuridine was purchased from the Aldrich Chemical Co., Metuchen, NJ, and SQ11725 (nadolol) was supplied by the Squibb Institute for Medical Research, Princeton, NJ.

Results and discussion

The reports by Zinder and Greenberg [14, 15] of β-adrenergic modulation of BAM cell secretion suggested that secreted EPI could exert a feed-forward potentiation of secretion by stimulating adenylate cyclase. Our initial experiments confirmed the finding of Zinder and Greenberg that propranolol inhibits CCh-induced CA secretion in BAM cells. Both *d*-propranolol and *l*-propranolol depressed the efficacy but not the potency of CCh for CA secretion (data not shown). Since both isomers of propranolol inhibited secretion, we examined the dose dependency of *d*- and *l*-propranolol on maximal CCh-

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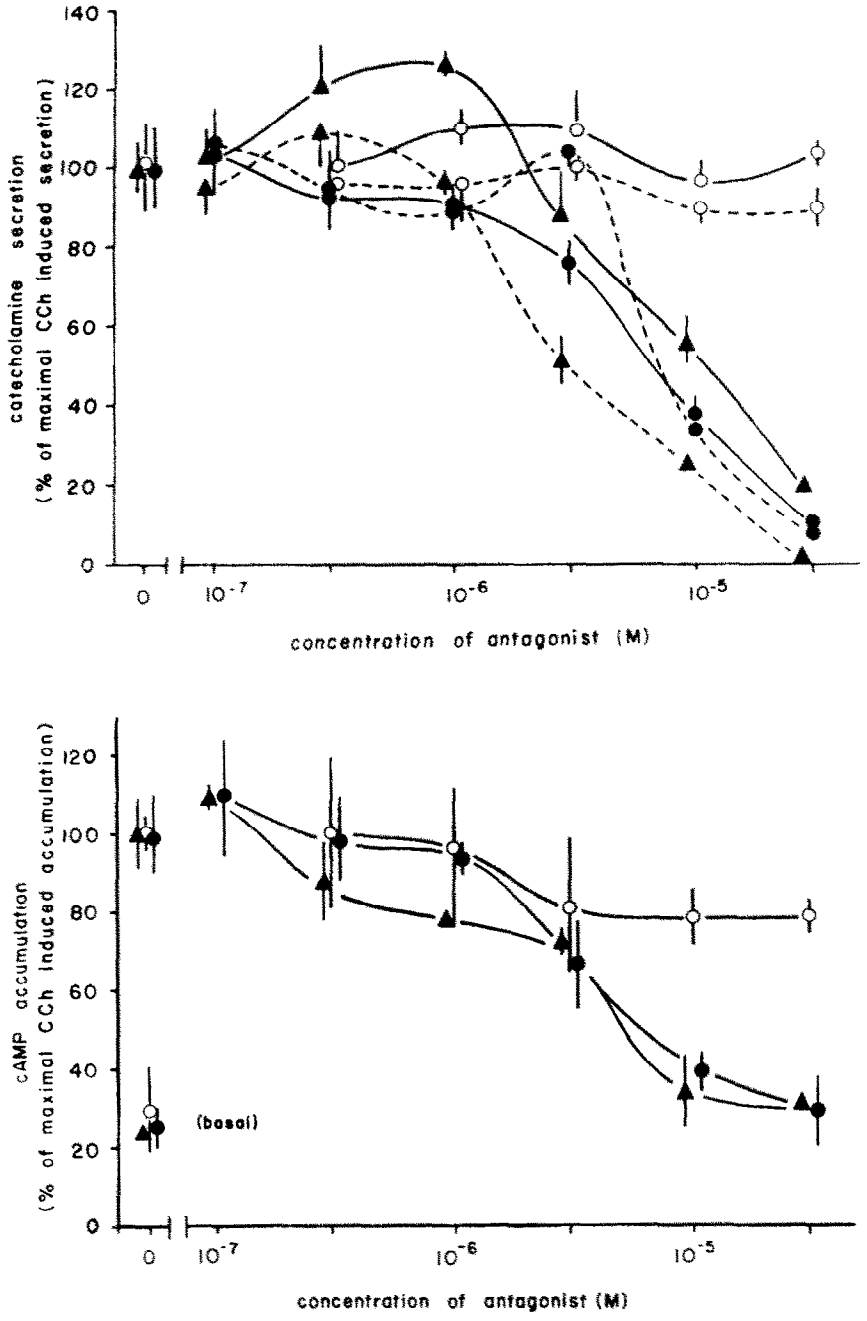

Fig. 1. Effects of β-adrenergic antagonists on BAM cell secretion and cellular cAMP levels. BAM cells were preincubated in buffer B for 10 min at 25° with various concentrations of β-adrenergic antagonists. After 10 min, the preincubation buffer was replaced with fresh buffer B containing β-adrenergic antagonist and 3×10^{-4} M CCh for a 10-min secretion period. The secretion buffer was then removed and assayed for CAs. Cellular CAs and cAMP were extracted with 0.1 N HCl. Secreted CAs were calculated as the percent of total CA and were expressed as the percent of the maximal response to 3×10^{-4} M CCh in each experiment. Typical total CA levels were in the range of 5–6 μg NE and 2.5–3.5 μg EPI/10^6 cells. Maximal CCh-stimulated release ranged from 25 to 40% for NE and 14 to 27% for EPI. Basal cAMP concentration ranged from 0.8 to 1.0 pmoles/10^6 cells and from 2.5 to 3.4 pmoles/10^6 cells after CCh stimulation. Values represent the means ± S.E.; N = 3 for *d*-propranolol and nadolol; N = 2 for *l*-propranolol. P values are indicated in the text. Key: (A) NE (solid lines) and EPI (dashed lines) secretion in response to 3×10^{-4} M CCh in the presence of increasing concentrations of *l*-propranolol (▲) *d*-propranolol (●) and nadolol (○). (B) Cellular cAMP accumulation in response to 3×10^{-4} M CCh in the presence of increasing concentrations of *l*-propranolol (▲), *d*-propranolol (●) and nadolol (○). [Values marked (basal) in Fig. 1B represent cAMP levels in the absence of CCh.]

induced secretion. The results are shown in Fig. 1A. Both stereoisomers produced identical dose-dependent inhibitions of EPI and NE secretion at concentrations greater than 10^{-6} M. At 10^{-5} M, *d*-propranolol inhibited NE and EPI secretion by 62% ($P < 0.01$) and 66% ($P < 0.01$) respectively; 10^{-5} M *l*-propranolol inhibited NE and EPI by 44% ($P < 0.01$) and 75% ($P < 0.01$) respectively. In other β-adrenergic systems, *l*-propranolol has been found to be 100 times more potent as an antagonist than *d*-propranolol [22].

BAM cells responded to CCh stimulation with a rise in cellular cAMP accumulation (Fig. 1B). CCh stimulation of cAMP accumulation varied between preparations from 20 to 800% increase over control. CCh-stimulated cAMP levels were well maintained for up to 20 min following stimulation (the earliest time measured was 5 min). Both *d*- and *l*-propranolol produced dose-dependent inhibition of CCh-stimulated cAMP accumulation with maximal inhibition occurring at 10^{-5} M ($P < 0.02$ for *l*-propranolol and $P < 0.01$ for *d*-propranolol with respect to CCh stimulation). The inhibitory effects of *d*- and *l*-propranolol on CA secretion and on cAMP accumulation could be explained either by β-receptor blockade or by membrane stabilization. The latter would decrease inward Ca^{2+} flux and possible calmodulin activation of adenylate cyclase [23]. β-Receptor blockade seemed to be an unlikely explanation based on the high concentrations of propranolol that were required and the lack of stereospecificity. To distinguish between these mechanisms, we tested the effects of SO11725 (nadolol) on CCh-induced secretion and cAMP accumulation. Nadolol is a potent non-selective β-adrenergic antagonist which does not possess membrane stabilizing activity [24]. Nadolol in concentrations from 3×10^{-7} M to 3×10^{-5} M had no effect on CCh-induced secretion (Fig. 1A, $P > 0.20$ at 3×10^{-5} M) and only a slight effect on cAMP accumulation (Fig. 1B, $P < 0.05$ at 3×10^{-5} M). These results indicated that the inhibitory activity of propranolol on BAM cell secretion probably was not due to blockade of β-adrenergic receptors.

Membrane stabilization would not, however, explain the reported CA secretory response to INE. Since INE is a substituted catecholamine, it was tested alone to see whether it would form a fluorescent trihydroxyindole derivative in our CA assay. INE was found to yield greater fluorescence than either EPI or NE and, therefore, could interfere with CA analysis. We measured the effects of INE on CA secretion by using HPLC/EC. INE was easily separated from EPI, dopamine and NE due to its considerably longer retention time on the C_{18} column. The effects of 10^{-5} M INE on basal and CCh-stimulated CA release are shown in Fig. 2A. INE neither stimulated secretion by itself nor potentiated maximal CCh-induced secretion. INE (10^{-5} M) had no effect on either basal ($P > 0.20$) or CCh-stimulated ($P > 0.20$) accumulations of cellular cAMP (Fig. 2B). No concentration of INE studied (10^{-8} to 10^{-5} M) had any effect on CA secretion or cAMP accumulation (only data for 10^{-5} M are shown).

The addition of exogenous cAMP in the form of 8-Bromo-cAMP potentiated the maximal secretory response to CCh ($P < 0.05$ for both NE and EPI) without altering basal secretion ($P > 0.05$ for EPI and NE) (Fig. 3). 2'-, 3'-cAMP, which is not an activator of cAMP-dependent protein kinase [25], had no effect on CCh-stimulated secretion ($P > 0.05$ for both NE and EPI) but did inhibit basal NE release ($P < 0.05$). While we were not able to reproduce the stimulatory effect of cAMP on basal secretion reported by Zinder and Greenberg [14], our results are consistent with a modulatory role for cAMP in BAM cell secretion. The pheochromocytoma cell line, PC-12, has also been shown to respond to forskolin-induced elevation in cAMP levels with a potentiation of depolarization-induced secretion without an effect on basal release [26].

In summary, our data suggest that β-adrenergic receptors are not involved in the BAM cell response to either propranolol or INE. The ability of propranolol to inhibit CCh-stimulated BAM cell secretion and cAMP accumulation reflected membrane stabilization and not β-adrenergic receptor antagonism. Nadolol, which lacks the membrane-stabilizing property of propranolol and is a potent β-adrenergic antagonist, failed to affect either CCh-stimulated secretion or cAMP accumulation. Using HPLC detection of CAs, we found no evidence of either INE stimulation of secretion or potentiation of CCh-induced secretion. INE also had no effect on cAMP accumulation. Our data support the contention that cAMP is involved in the regulation of BAM cell secretion. Such an involvement

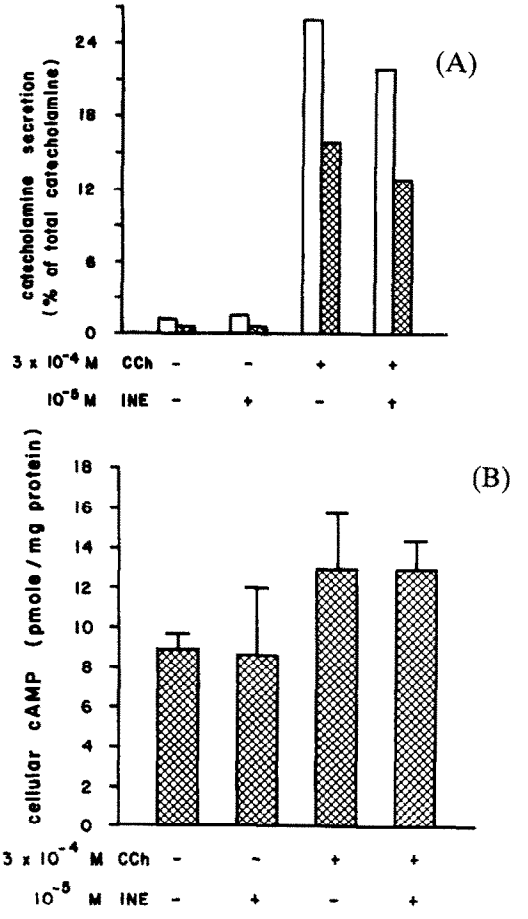

Fig. 2. Effects of INE on BAM cell secretion and cAMP accumulation. BAM cells were preincubated for 10 min at 25° in the presence (+) or absence (−) of 10^{-5} M INE and then were incubated for another 10 min in fresh buffer in the presence or absence of 10^{-5} M INE and in the presence or absence of 3×10^{-4} M CCH. Secretion buffer was removed and frozen. Cellular cAMP and CAs were extracted with 0.1 N HCl. Aliquots of the acid extracts were frozen and, together with the frozen secretion buffers, were assayed for CAs by HPLC/EC. Representative total NE and EPI values were 8 and 2.7 μg/10^6 cells respectively. Representative cAMP levels were: basal 0.8 pmoles/10^6 cells, and CCh-stimulated, 1.3 pmoles/10^6 cells. (A) NE (open bars) and EPI (hatched bars) secretion in the presence (+) or absence (−) of 10^{-5} M INE and 3×10^{-4} M CCh (N = 1). (B) Cellular cAMP accumulation in the presence (+) or absence (−) of 10^{-5} M INE. cAMP values represent the means ± S.E. with N = 2. P values are given in the text.

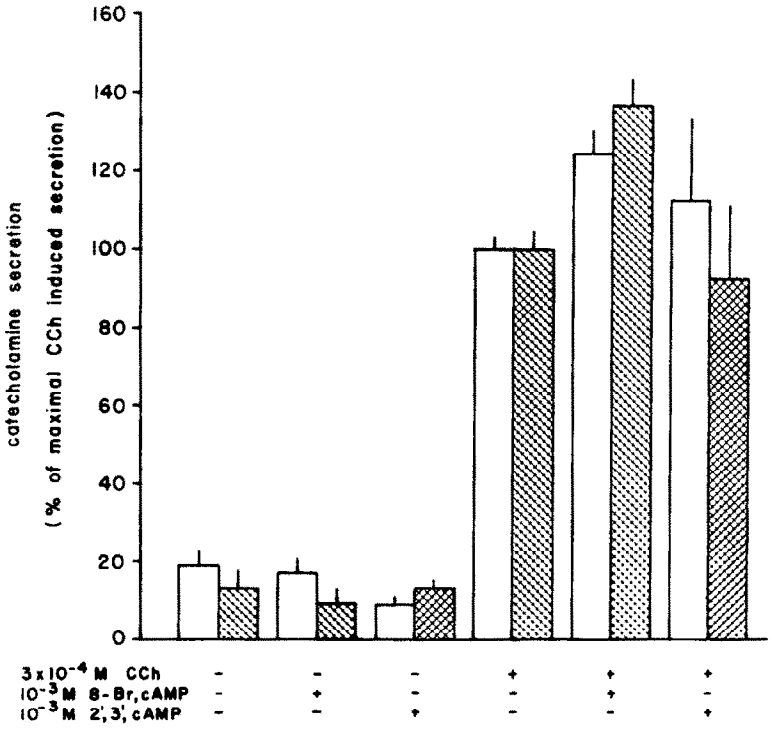

Fig. 3. Effects of exogenous cAMP analogues on BAM cell secretion. BAM cells were preincubated for 10 min at 25° in the presence (+) or absence (−) of 10^{-3} M 8-Bromo-cAMP or 2′,3′-cAMP. The preincubation buffer was assayed for basal CA secretion. The BAM cells were incubated for a second 10 min in the presence (+) or absence (−) of the cAMP analogues and 3×10^{-4} M CCh. The secretion buffer was then removed and assayed for CA content. Cellular CA was extracted with 0.1 N HCl. Secreted CAs were calculated as the percent of total CAs and expressed here as the percent of the response to 3×10^{-4} M CCh in each experiment. Representative basal NE and EPI totals were 10 and 6.5 $\mu g/10^6$ cells respectively. Maximal CCH stimulated 24% of total NE and 15% of total EPI. NE (open bars) and EPI (hatched bars) values represent the means ± S.E. of duplicate determinations.

is consistent with known actions of cAMP in other secretory systems.

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

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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).