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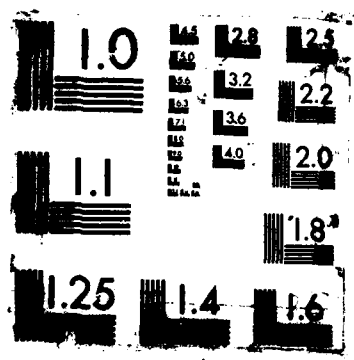
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CELLULAR ACTIONS AND INTERACTIONS OF ANTICHOLINESTERASES
AND THEIR ANTIDOTES IN MAMMALIAN AUTONOMIC NEURONS

Annual Summary Report

July 29, 1985

N.J. Dun, Ph.D.

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3133

Loyola University
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Summary

The major objectives of this study are (1) elucidation of the cellular mechanism of the facilitatory and blocking effects of organophosphorus anti-cholinesterase (anti-ChE) agent, diisopropylfluorophosphate (DFP) on sympathetic neurons and on ganglionic transmission; and (2) clarification of the site and mechanism of action of a cholinesterase (ChE) reactivator, pyridinealdoxime (2-PAM) on cholinergic transmission. Isolated rabbit superior cervical ganglia or guinea pig inferior mesenteric ganglia were used in this study. Intracellular recordings were obtained from neurons of the isolated sympathetic ganglia by means of glass microelectrodes. DFP, 2-PAM and other agents were applied to the ganglia either by superfusion in known concentrations or by pressure ejection from a micropipette containing appropriate agents.

DFP exerted a dose-dependent action on nicotinic and muscarinic transmission of the sympathetic neurons. At concentrations of 10 μ M or less, DFP increased the amplitude as well as the duration of the fast excitatory postsynaptic potential (f-epsp) which is nicotinic in nature. The nicotinic acetylcholine (ACh) depolarization induced by ACh applied by pressure ejection was likewise increased. On the other hand, DFP at concentrations of 0.1 mM or higher consistently and reversibly depressed f-epsp's and ACh depolarizations. The effects of DFP on depolarizations induced by carbamylcholine (CaCh), a ChE resistant cholinergic agonist, were concentrations dependent. DFP at concentrations of 10 μ M or less did not noticeably change the amplitude or duration of CaCh-induced depolarizations, whereas, the latter were reversibly blocked by DFP at concentrations of 0.1 mM or higher.

The effects of DFP on muscarinic transmission were also concentrations dependent. DFP at concentrations of 10 μ M or less increased the amplitude and duration of the slow excitatory postsynaptic potential (s-epsp) which is muscarinic in nature as well as the depolarization induced by methacholine (MeCh), a specific muscarinic agonist which is slowly hydrolyzed by ChE. On the other hand, DFP at concentrations of 0.1 mM or higher reversibly blocked the s-epsp as well as MeCh depolarization.

At concentrations of 1 mM or lower and 10 mM or higher, 2-PAM increased and decreased the nicotinic depolarizations whether evoked synaptically or by pressure ejections of ACh. Thus, the results provide evidence for the first time that DFP and 2-PAM exerted an acetylcholinesterase (AChE) dependent facilitation and an AChE independent inhibition of nicotinic transmission. It is ~~therefore~~ concluded that contrary to the long standing concept that DFP and 2-PAM affect cholinergic transmission solely by inhibiting and reactivating junctional ChE, respectively, these compounds exert direct actions on cholinergic nicotinic and muscarinic receptors/ion-channel complex that are independent of the status of junctional ChE activity.

In addition to these two major studies, we found that d-tubocurarine (d-Tc, 10-100 μ M) and prostaglandin ~~E₁~~ (PGE₁, 10-100 nM) reversibly and dose-dependently suppressed the spike after-hyperpolarization (AH) of rabbit and guinea pig sympathetic ganglion cells. The ability of these two compounds to suppress spike AH is interesting as they may increase the membrane excitability of sympathetic neurons and other central neurons.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals." prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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Report

I. Introduction

While phosphorylation of the active site of cholinesterases (ChE) and the resulting accumulation of acetylcholine (ACh) is clearly involved in many aspects of the pharmacological actions of anticholinesterase (anti-ChE) agents, including organophosphorous (OP) compounds (1,2), a number of studies suggest that these pharmacological or toxicological actions may not be entirely related to their anti-ChE activity. For example, anti-ChE agents block ganglionic transmission (3,4,5,6,7,8), yet the concentrations of anti-ChE agents needed to block ganglionic transmission do not seem to be closely related to the concentrations that produce inhibition of ganglionic acetylcholinesterase (AChE) (9,10). Accordingly, several investigators suggested that the ganglionic or neuromyal action of anti-ChE agents depend on their direct actions (9, 11, 12, 13). Results obtained during the first year of our studies supported by this contract show that anti-ChE agents of carbamate type, physostigmine and neostigmine, depressed nicotinic transmission by a direct action on nicotinic receptors and/or ion channel complex (14). During this reporting year, the effects of diisopropylfluorophosphate (DFP), an OP type of anti-ChE agent on sympathetic neurons and on ganglionic transmission were studied. The hypothesis was that OP anti-ChE agents similar to the carbamate type may depress ganglionic transmission by a direct action that is independent of ChE inhibition.

The second major study during the past year concerns the effects of pralidoxime (2-PAM), a prototype of ChE reactivator (15), on ganglionic transmission. The aim was to evaluate the effectiveness of 2-PAM in reversing or preventing the effects of OP agents on ganglionic transmission. Surprisingly, our results show that 2-PAM has a number of actions that appear to be independent of reactivation of ChE.

A brief summary of the electrophysiological and pharmacological properties of ganglionic transmission is intended to facilitate understanding of the results obtained here.

Results from these and other laboratories have established over the past few years that synaptic transmission of vertebrate sympathetic ganglia is a complex, multi-transmitter-mediated event consisting of several post-synaptic potentials of opposing polarities and time courses ranging from a few milliseconds to minutes (16,17,18,19).

Three of these responses are depolarizing (or excitatory) potentials. When the principal transmitter, ACh, is liberated presynaptically, it acts on postsynaptic nicotinic and muscarinic receptors and generates, respectively, the fast and slow excitatory postsynaptic potentials (f-epsp and s-epsp). In addition, in some ganglia, a long-lasting depolarization that is noncholinergic in nature, as it is insensitive to nicotinic and muscarinic antagonists, appears following the pre-synaptic stimulation. Current pharmacological, biochemical and ultrastructural data suggest that this non-cholinergic response is generated in mammalian pre-vertebral ganglia by either substance P, an undecapeptide (20,21) or serotonin (22).

In addition to these three epsp's, the sympathetic ganglia exhibit the slow inhibitory postsynaptic potential (s-ipsp). The s-ipsp is generated either monosynaptically by a muscarinic action of ACh, or disynaptically, by dopamine released from a specialized interneuron, the small intensely fluorescent (SIF) cell, which is first activated by the muscarinic action of ACh (16,17,18,23).

The excitatory and inhibitory responses described so far interact to provide for a sensitive control of ganglionic transmission. This control is further refined via release of additional bioactive substances. Indeed, it was established that these substances--which include additional peptides such as enkephalins, amino acids such as gamma-aminobutyric acid (GABA), prostaglandins, catecholamines and indolamines--are endogenous to the ganglia, and that their exogenous application effectively alters membrane potentials and/or transmitter release. These findings strongly imply that these substances play a role in the modulation of ganglionic transmission (17,18,19).

II. Methods and Materials

Adult male rabbits (1.5-2 Kg) or guinea pigs (25-300 g) were used in this study. The animals were sacrificed by overdose of pentobarbital, and the superior cervical ganglia from the rabbits or the inferior mesenteric ganglia from the guinea pigs were rapidly excised together with their preganglionic nerve trunks and transferred to the recording chamber. The ganglia were superfused with Krebs solution of the following composition (in millimolar concentrations): NaCl, 117; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 25; NaHPO₄, 2.2; and glucose, 11.5, the solution was gassed with 95% O₂ and 5% CO₂ and the temperature of the solution was maintained at 34±0.5°C. The intracellular recording techniques have been described in detail (14). In brief, intracellular recordings were obtained from neurons by using fiber-containing glass capillaries filled with 3 M KCl that had resistance of 35-60 megohms. The preganglionic nerve trunk was drawn into a suction electrode for orthodromic stimulation. Nicotinic f-epsp's were induced by electrical stimulation of the cervical sympathetic trunk at low frequency (0.1-0.3 Hz). ACh potentials were evoked by pressure ejection of ACh from a micropipette positioned above the ganglion cell from which intracellular recordings were made. ACh was discharged from the micropipette by nitrogen of various pulse duration (5-10 ms).

In experiments of the effects of DFP or 2-PAM on muscarinic transmission, the ganglia were superfused continuously with a Krebs solution containing d-Tc (50 µM) to suppress the nicotinic transmission of the ganglia. Repetitive nerve stimulation at the frequency of 20-30 Hz for 1-3 sec was used to elicit the muscarinic synaptic responses. DFP and 2-PAM were dissolved in Krebs solution and applied to the ganglia in known concentrations by superfusion.

The following compounds were used: Acetylcholine chloride, atropine sulfate, PGE₁, d-tubocurarine chloride, and diisopropylfluorophosphate were purchased from Sigma Co., 2-pyridinealldoxime methyl methanesulfonate was obtained from Aldrich Chemical Co.

III. Results

1. Effects of DFP on nicotinic transmission

The dose-effect relationships of DFP were examined on f-epsp's which are mediated by a nicotinic action of ACh released synaptically (16,17) and on nicotinic

depolarizations induced by pressure ejection of ACh or CaCh. These experiments were carried out in the presence of atropine ($1\text{ }\mu\text{M}$) to ensure the elimination of muscarinic action of ACh and CaCh.

A. Facilitatory effects of DFP on f-epsp's

The effects of DFP on the f-epsp's were clearly concentrations dependent. At concentrations of $1\text{--}10\text{ }\mu\text{M}$, DFP applied for 5 min consistently and reversibly increased the amplitude of subthreshold f-epsp's, leading to spike discharges in all 12 cells examined; a representative experiment is shown in Fig. 1B. The potentiating effect was long lasting; a 1-2 hr washing period with Krebs solution was generally needed for the response to return to the control level (Fig. 1B). A quantitative determination of the potentiation of DFP on f-epsp's was difficult as the latter invariably reached threshold and fired action potentials in the presence of DFP (Fig. 1B).

B. Blocking action of DFP on f-epsp's

In contrast to its enhancing effect at lower concentrations, DFP at concentrations of 0.1 mM and 1 mM completely blocked f-epsp's in 25 of the 28 ganglion cells investigated. An experiment in which DFP (1 mM) reversibly blocked the f-epsp's of a rabbit ganglion cell is shown in Fig. 1A. The blocking effect of DFP generally developed in 3-5 min following the addition of DFP and reversed in about 30 min after discontinuation of DFP superfusion. It is interesting in this respect to point out that f-epsp's recovered much faster from the blocking effect of DFP as compared with that of the enhancing action.

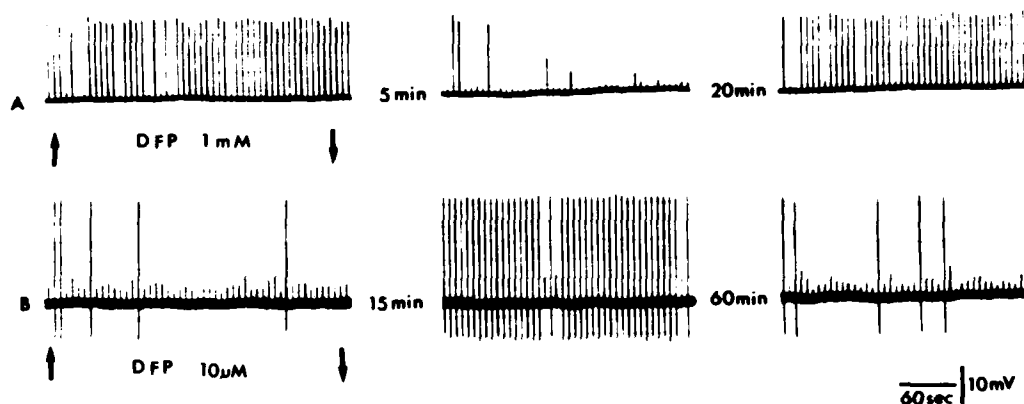


Fig. 1 Potentiating and blocking effects of DFP on f-epsp's evoked in two superior cervical ganglion cells. A: DFP (1 mM) applied between two arrows completely and reversibly blocked the f-epsp's (upward deflections) elicited by low frequency stimulation of cervical sympathetic nerve trunk. The amplitude of f-epsp's recovered to near control level 25 min after discontinuation of DFP superfusion. B: potentiating action of DFP ($10\text{ }\mu\text{M}$) on subthreshold f-epsp's evoked by low frequency electrical stimulation of cervical sympathetic nerve trunk. Following DFP superfusion, subthreshold f-epsp's reached threshold and fired action potentials (middle panel). The responses returned to control level more than 1 hr after washing with Krebs solution.

2. Effects of DFP on nicotinic depolarizations

The purpose of these experiments was to evaluate whether DFP depresses and enhances nicotinic transmission by altering the sensitivity of postsynaptic membrane nicotinic receptors to ACh or affecting release of ACh. For this series of experiments, the effects of DFP on membrane depolarizations induced by pressure ejection of ACh or CaCh were examined. Atropine (1 μ M) was routinely present in the perfusing Krebs solution to block the muscarinic effect of ACh and CaCh.

A. Nicotinic depolarizations induced by ACh

Similar to the effects of DFP on f-epsp's evoked synaptically, DFP increased and blocked nicotinic depolarizations induced by ACh in a concentration dependent manner (Table 1). One experiment in which DFP (10 μ M) increased the amplitude and duration of ACh induced depolarization is illustrated in Fig. 2A. The facilitatory action was fairly long lasting. Generally, one hour or more of washing with Krebs solution was needed for the ACh depolarization to return to control level. On the other hand, DFP blocked the ACh depolarization at concentrations of 0.1 mM or higher (Table 1). One experiment where DFP (1 mM) completely blocked the ACh induced depolarization is depicted in Fig. 2A.

Table 1. Effects of DFP on ACh depolarizations induced by pressure ejection of ACh in rabbit superior cervical ganglion cells.

ACh Depolarizations		
DFP	% change of amplitude mean $\pm$ S.E.M.	% change of duration mean $\pm$ S.E.M.
10 μ M (n=4)	+43 $\pm$ 8%*	+52 $\pm$ 7%*
0.1 mM (n=6)	-66 $\pm$ 9%**	-42 $\pm$ 9%**
1 mM (n=8)	-93 $\pm$ 2%**	-91 $\pm$ 5%**

* statistically significant ($p < 0.05$)

** statistically significant ($p < 0.01$)

B. Nicotinic depolarizations induced by CaCh

DFP at the concentration of 10 μ M or less caused no discernible change of the membrane depolarization induced by pressure ejection of CaCh (Table 2). One such experiment is shown in Fig. 3 (right panel). On the other hand, DFP at concentrations of 0.1 mM or higher depressed or blocked the CaCh induced depolarizations in all 6 cells studied (Table 2). A representative experiment is shown in Fig. 3 (left panel).

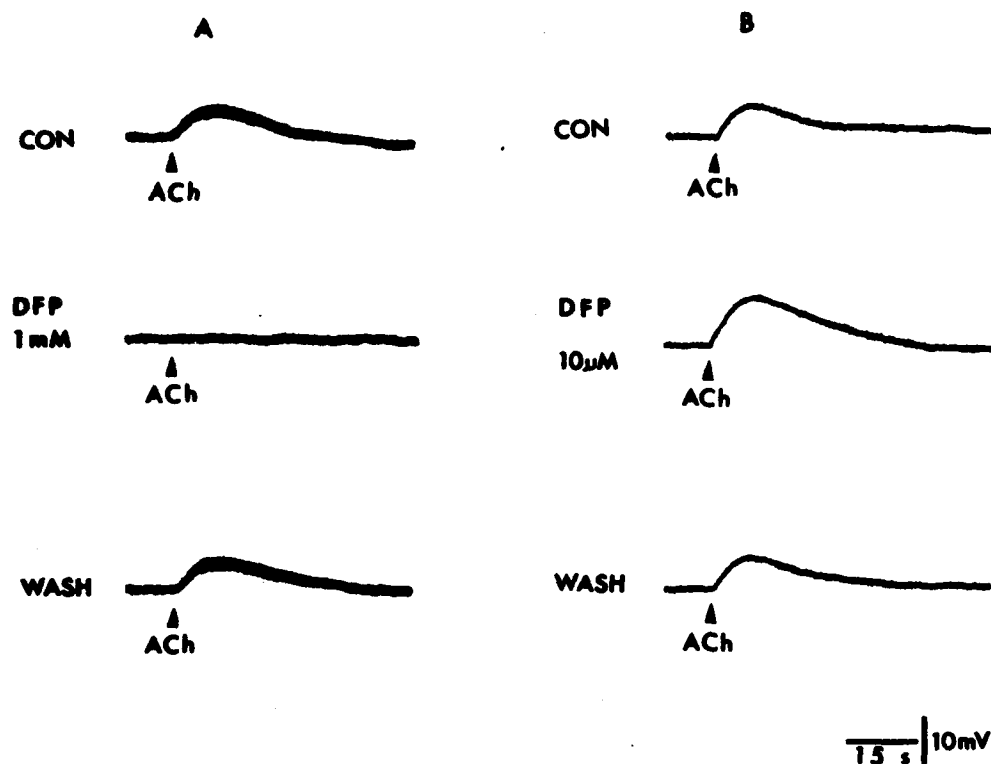


Fig. 2 Potentiating and blocking effects of DFP on nicotinic ACh depolarizations induced by pressure ejection of ACh in two rabbit superior cervical ganglion cells in the presence of atropine (1 μ M). A: Control nicotinic ACh depolarization was evoked by pressure ejection of ACh (arrowhead, 10 ms pulse duration). Following superfusion of DFP (1 mM), pressure ejection of ACh (arrowhead) caused no detectable membrane depolarization. ACh depolarization returned to control level after a period of 20 min wash with Krebs solution. B: ACh depolarization evoked by pressure ejection of ACh (arrowhead) was increased by about 50% following DFP (10 μ M) superfusion. ACh depolarization returned to control level about 50 min after washing with Krebs solution (bottom tracing).

Table 2. Effects of DFP on CaCh depolarizations induced by pressure ejection of CaCh in rabbit superior cervical ganglion cells.

DFP	CaCh depolarizations	
	% change of amplitude mean $\pm$ S.D.	% change of duration mean $\pm$ S.D.
10 μ M (n=4)	+7 $\pm$ 3%	+4 $\pm$ 2%
1 mM (n=5)	-92 $\pm$ 7%*	-95 $\pm$ 5%*

* statistically significant ($p < 0.01$)

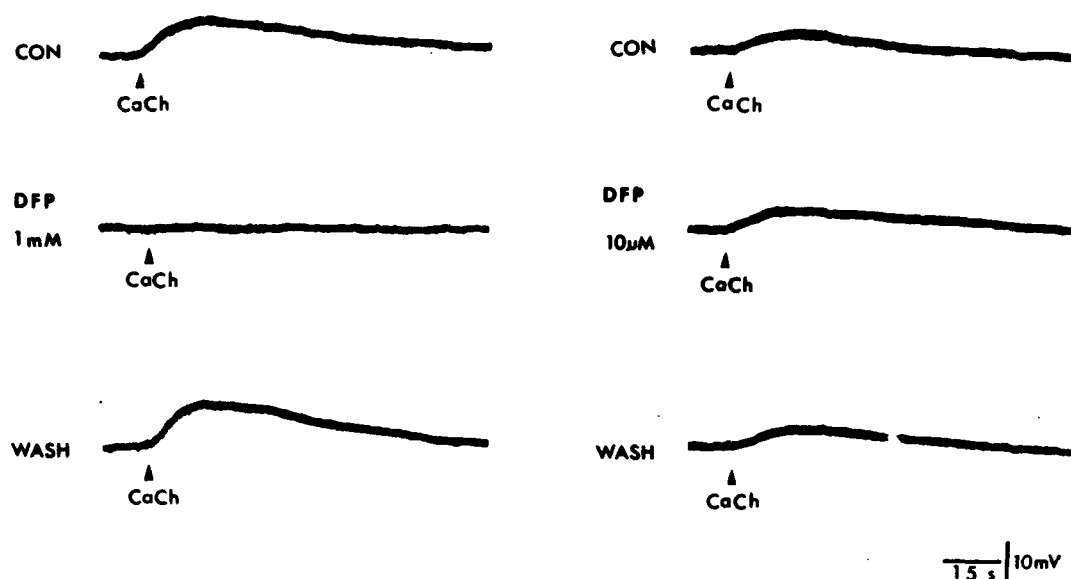


Fig. 3. Effects of DFP on nicotinic depolarization evoked by CaCh in a rabbit superior cervical ganglion cell in the presence of atropine ($1 \mu\text{M}$). DFP ($10 \mu\text{M}$) caused no appreciable change of the nicotinic depolarization induced by CaCh (arrowhead). Following superfusion of DFP (1 mM), pressure ejection of CaCh elicited no membrane depolarization. CaCh depolarization returned to control level after a period of 30 min wash.

3. Effects of DFP on membrane electrical properties

DFP at the concentration as high as 1 mM did not cause a significant change of the resting membrane potential and input resistance in the majority of sympathetic neurons tested. However, DFP (0.1 – 1 mM) caused a small ($\leq 5 \text{ mV}$) depolarization in 29 of the 130 cells sampled. The DFP induced depolarizations exhibited several interesting features. First, the DFP depolarization was generally not associated with any detectable increase or decrease of membrane resistance. Second, the depolarization showed no tachyphylaxis, i.e., the depolarization persisted as long as DFP was in the perfusing solution. Finally, pretreating the ganglia with low Ca solution or nicotinic and muscarinic antagonists did not alter the DFP induced depolarizations. One experiment in which DFP application caused a slow depolarization and blocked of nicotinic transmission is shown in Fig. 4. It must however be stressed that there is no clear-cut correlation between DFP induced depolarization and synaptic blockade as in the majority of cells DFP caused little or no change in membrane potentials.

4. Effects of DFP on muscarinic transmission

Neurons in the rabbit superior cervical ganglia in response to preganglionic nerve stimulation generate, in addition to the initial f-epsp, a slow excitatory postsynaptic potential (s-epsp) which is mediated by a muscarinic action of ACh (16,17). Interestingly, DFP facilitated and blocked muscarinic transmission in a concentration-dependent manner.

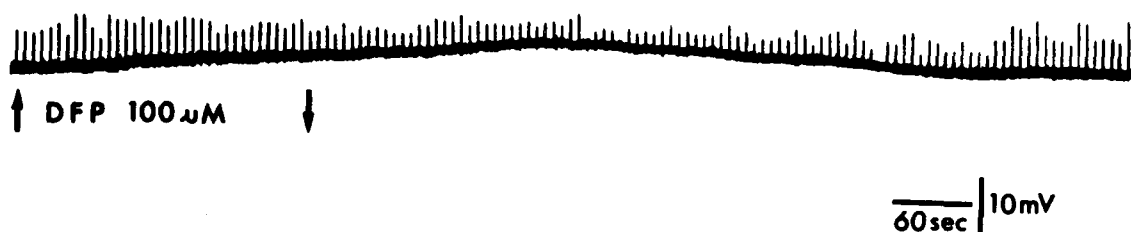


Fig. 4. Synaptic depression and membrane depolarization caused by DFP in a rabbit superior cervical ganglion cell. Superfusion of DFP (100 μ M) caused a slow depolarization and concomitant depression of f-epsp's (small upward deflections).

A. Facilitation and depression of s-epsp

S-epsp's were elicited in neurons of the rabbit superior cervical ganglia by a short train of stimuli applied to the cervical sympathetic trunk (10-20 Hz, 1-2 sec) in the presence of d-Tc (50 μ M) which suppresses the initial f-epsp's. Typically, the s-epsp shows an amplitude of 5-20 mV and a duration of seconds (16,17,18). DFP at concentrations of 10 μ M or less facilitated the s-epsp's, whereas, it depressed the responses when the concentration was raised to 1 mM or higher (Table 3). An experiment in which DFP (10 μ M) increased the amplitude and prolonged the duration of s-epsp is shown in Fig. 5 (lower tracings). The facilitation was long lasting and 2-3 hr of washing with Krebs solution was generally required for the response to return to control level (Fig. 5). In the same neuron, DFP at higher concentration (1 mM) reversibly depressed the s-epsp (Fig. 5, upper tracings).

Table 3. Effects of DFP on muscarinic s-epsp's evoked in rabbit superior cervical ganglion cells.

	Muscarinic s-epsp's					
	Control (n=7)	DFP 10 μ M	% change	Control (n=8)	DFP 1 mM	% change
Amplitude, mV	3.2 $\pm$ 0.2	9.8 $\pm$ 1.2	+196.7 $\pm$ 56.8*	6.8 $\pm$ 1.2	2.3 $\pm$ 1.1	-82 $\pm$ 11.1%**
Duration, sec.	26.0 $\pm$ 4.0	54.0 $\pm$ 8.7	+108.3 $\pm$ 15.4%**	36.7 $\pm$ 2.1	13.0 $\pm$ 3.1	-75.0 $\pm$ 7.8%**

* Statistically significant ($p < 0.05$)

** Statistically significant ($p < 0.01$)

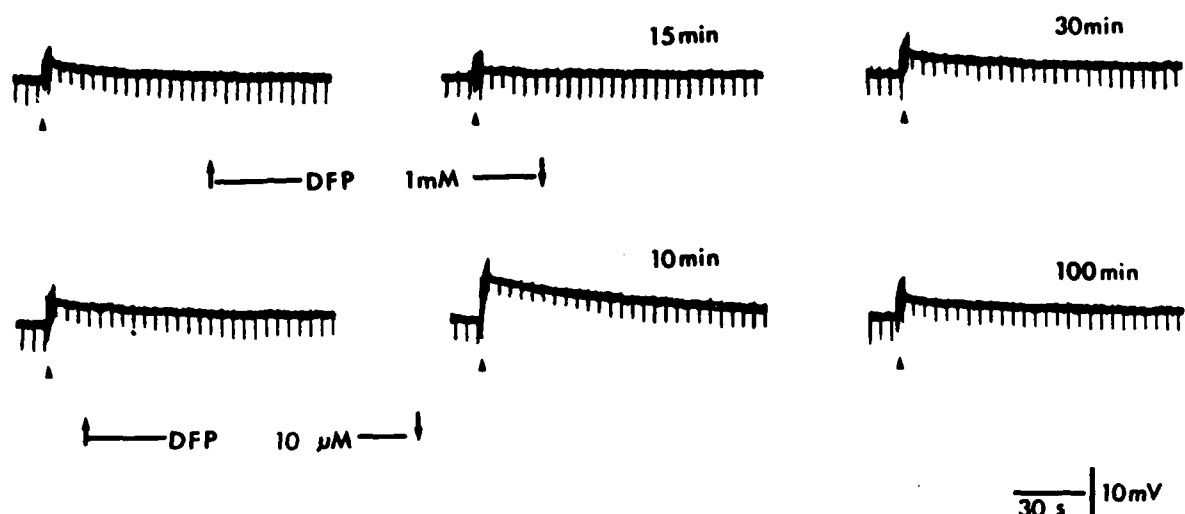


Fig. 5 Potentiation and depression of s-epsp's by DFP in a rabbit superior cervical ganglion cell. S-epsp's were induced by repetitive stimulation of cervical sympathetic nerve trunk (30 Hz, 2 sec, arrowhead). In this experiment, d-Tc (50 μ M) was present in the Krebs solution to suppress the f-epsp's. DFP (1 mM) superfusion blocked the s-epsp; the latter recovered to control level 30 min after washing with Krebs solution. The s-epsp was markedly increased following the superfusion of DFP (10 μ M). The s-epsp returned to control level after a prolonged washing period. The recordings were taken from the same ganglion cell.

B. Facilitation and depression of muscarinic depolarizations induced by methacholine (MeCh)

This series of experiments was designed to evaluate whether DFP affects muscarinic transmission by a pre- or postsynaptic mechanism or both. For these experiments, muscarinic depolarizations were evoked by pressure ejection of MeCh onto the ganglion cells. DFP potentiated and blocked muscarinic depolarizations induced by MeCh in a concentration dependent manner (Table 4). An experiment in which DFP at low concentration (10 μ M) increased and at high concentration (1 mM) blocked muscarinic depolarizations in the same ganglion cell is shown in Fig. 6.

Table 4. Effects of DFP on muscarinic depolarizations induced by MeCh in rabbit superior cervical ganglion cells.

MeCh depolarizations						
	Control (n=5)	DFP 10 μ M	% Change	Control (n=6)	DFP 1 mM	% Change
Amplitude, mV	5.1 $\pm$ 0.9	9.7 $\pm$ 2.6	+139.3 $\pm$ 36.5%**	5.3 $\pm$ 0.7	1.0 $\pm$ 0.6	-85.1 $\pm$ 9.8%**
Duration, sec	48.0 $\pm$ 9.6	61.4 $\pm$ 7.8	+32.1 $\pm$ 6.2%*	73.8 $\pm$ 5.7	3.7 $\pm$ 2.2	-84.3 $\pm$ 9.1%**

* Statistically significant ($p < 0.05$)

** Statistically significant ($p < 0.01$)

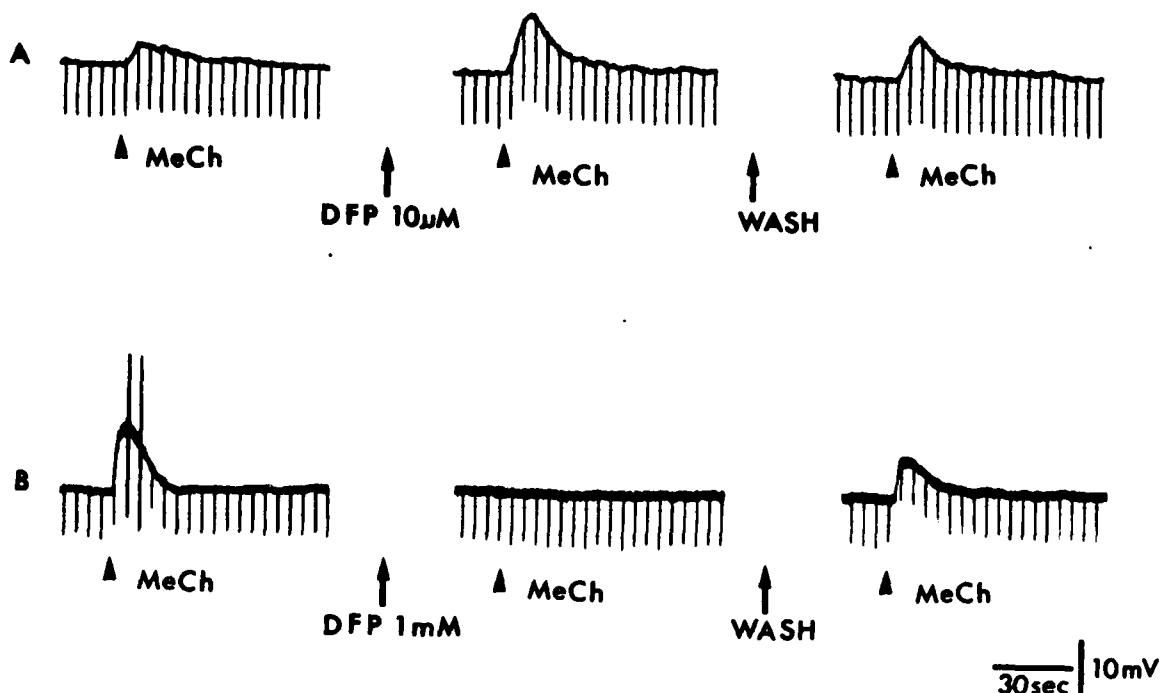


Fig. 6 Facilitation and depression of muscarinic depolarizations induced by DFP in a rabbit sympathetic ganglion cell. A: muscarinic depolarization was evoked by pressure ejection of MeCh (arrowhead, 20 ms pulse duration). MeCh depolarization was markedly enhanced following the superfusion of DFP (10 μ M). B: Following the superfusion of DFP (1 mM), pressure ejection of MeCh produced no membrane depolarization.

5. Unusual effects of DFP on muscarinic depolarization

An interesting and unusual effect of DFP on muscarinic depolarizations was noticed in 7 of the 32 cells; DFP converted the muscarinic depolarization into a hyperpolarizing response. In these 7 rabbit superior cervical ganglion cells MeCh applied by pressure ejection caused depolarization as expected. However, after DFP superfusion pressure ejection of MeCh now produced a hyperpolarization instead of a depolarization; one such experiment is shown in Fig. 7. This unusual effect of DFP was fully reversible after washing with Krebs solution. Furthermore, atropine effectively blocked the hyperpolarization induced by MeCh in the presence of DFP as seen in Fig. 7. Also interesting to point out is that the MeCh induced hyperpolarization under the influence of DFP was consistently associated with an increase in membrane resistance (Fig. 7).

6. Effects of 2-PAM on ganglionic transmission

The compound, 2-PAM is best known for its ability to reactivate phosphorylated ChE at neuromyal junctions (15). Results obtained here show that in addition to its reactivating properties, 2-PAM affects nicotinic transmission directly or indirectly.

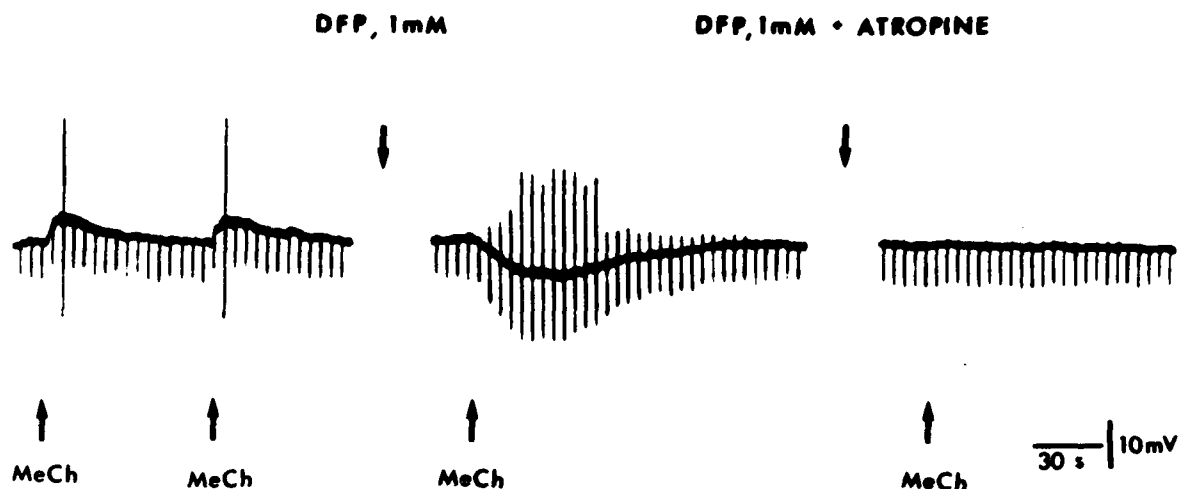


Fig. 7 Conversion of muscarinic depolarization to hyperpolarization by DFP in a rabbit superior cervical ganglion cell. Control responses consist of two muscarinic depolarizations induced by pressure ejections of MeCh (10 ms duration). Following the superfusion of DFP (1 mM) for 5 min, pressure ejection of MeCh now evoked a hyperpolarization associated with an increase in membrane input resistance as indicated by an increase in the amplitude of hyperpolarizing electrotonic potentials (small downward deflections). Atropine (1 μ M) completely blocked the effects of pressure ejection of MeCh.

A. Facilitation and depression of nicotinic transmission by 2-PAM

The effects of 2-PAM on nicotinic transmission were also concentration dependent. At concentrations of 1 mM or less, 2-PAM increased the amplitude of f-epsp's, leading to spike discharges in all 7 cells examined. A representative experiment is shown in Fig. 8: At the concentration of 10 mM, 2-PAM depressed f-epsp's. The mean reduction of the amplitude of f-epsp's in 10 cells studied was 82.5%. One of the experiments is shown in Fig. 9.

B. Facilitation and depression of nicotinic depolarizations by 2-PAM

In this series of experiments, the effects of 2-PAM on nicotinic depolarizations induced by pressure ejection of ACh were evaluated. Atropine (1 μ M) was present in the perfusing Krebs solution to ensure that the depolarization evoked by ACh was nicotinic.

Consistent with the data on nicotinic transmission, 2-PAM facilitated and depressed nicotinic ACh depolarizations dependent on the concentrations employed. At concentrations of 1 mM or less, 2-PAM increased the amplitude of ACh depolarizations as shown in Fig. 10. The mean increase was $63 \pm 16\%$ ($n=14$). At the concentration of 10 mM or higher, 2-PAM attenuated the nicotinic ACh depolarization in 6 of the 7 cells examined (Fig. 11); the mean decrease being $87 \pm 7\%$.

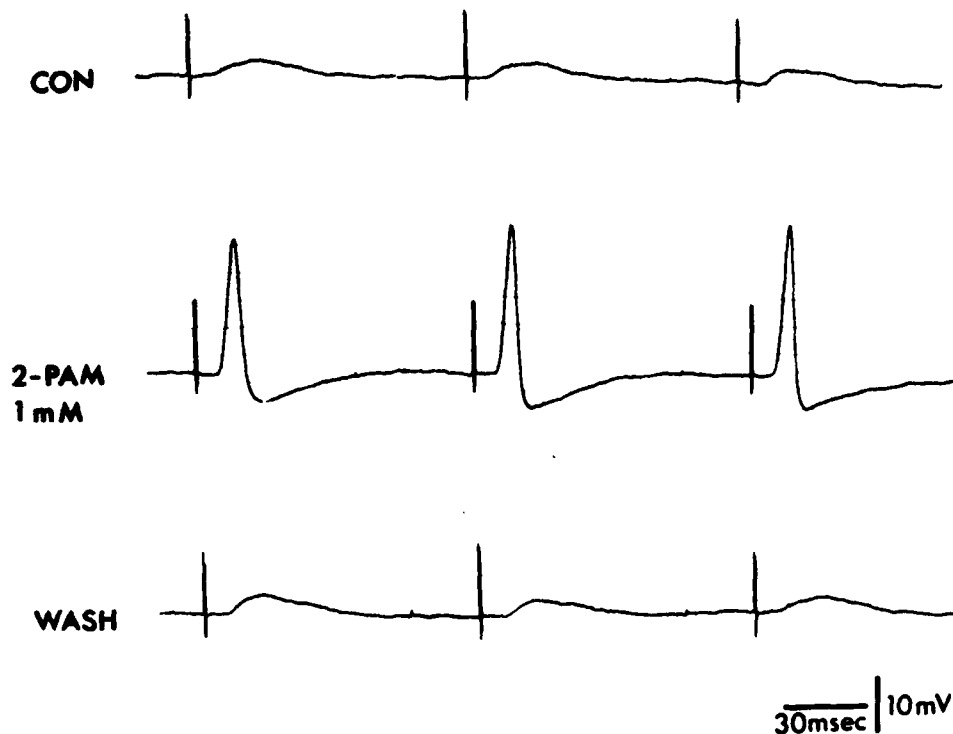


Fig. 8 Potentiation of f-epsp's by 2-PAM in a rabbit superior cervical ganglion cell. Control responses consist of three subthreshold f-epsp's. Superfusion of 2-PAM (1 mM) increased the amplitude of f-epsp's to a point where action potentials were initiated (middle tracing). F-epsp's recovered to control level 15 min after washing with Krebs solution.

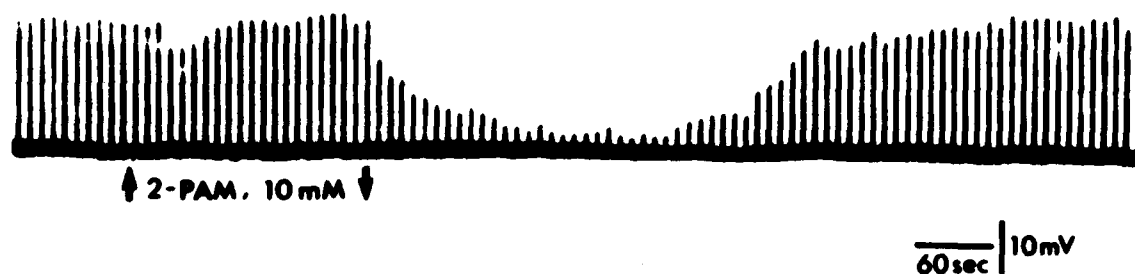


Fig. 9 Depression of f-epsp's by 2-PAM in a rabbit superior cervical ganglion cell. Upward deflections represent f-epsp's evoked by low frequency of stimulation of cervical sympathetic nerve trunk. While not affecting the membrane potential, superfusion of 2-PAM (10 mM) markedly reduced the amplitude of f-epsp's. The depressant action of 2-PAM was readily reversible.



Fig. 10 Increase of nicotinic ACh depolarizations by 2-PAM in a rabbit superior cervical ganglion cell. Pressure ejection of ACh (arrowheads) caused a brisk depolarization. Superfusion of 2-PAM markedly increased the amplitude of ACh depolarizations. The amplitude of ACh depolarizations returned to control level in about 10 min after discontinuation of 2-PAM superfusion.

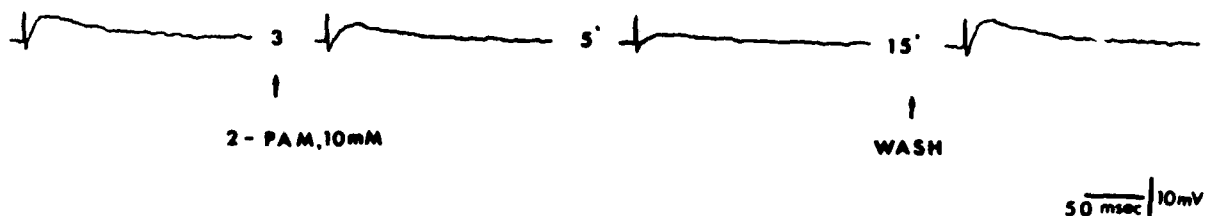


Fig. 11 Depression of nicotinic ACh depolarizations by 2-PAM in a rabbit superior cervical ganglion cell. Superfusion of 2-PAM for 3 min reduced the amplitude of ACh depolarization and it was nearly blocked after 5 min. The ACh depolarization returned to control level 15 min after washing.

7. Spike after-hyperpolarization (AH) and its suppression by d-Tc

During the course of our study on the effects of DFP on muscarinic responses, d-Tc (50 μ M) was routinely added to the Krebs solution to suppress nicotinic responses. What is interesting to note was that the spike AH is consistently reduced by d-Tc. This led to a more extensive study of the action of d-Tc on spike AH.

The spike potentials evoked in rabbit or guinea pig sympathetic neurons are followed by a prominent AH with an amplitude of 5-25 mV and duration of several hundred ms. The spike AH in the majority of rabbit and guinea pig sympathetic neurons consists of two distinct components: a fast decaying (AH_f) and a slow decaying (AH_s) component. Our results as well as results obtained in other

types of vertebrate and invertebrate neurons (24) show that AH_f is due to an increase of membrane conductance to K^+ (G_K), whereas the AH_s is probably caused by an increase of potassium conductance secondary to calcium influx (G_{K-Ca}).

In the concentrations of 10-100 μM , d-Tc quite selectively suppresses AH_s without affecting significantly the AH_f (Fig. 12). The depression of AH_s by d-Tc could be a result of either reduction of Ca influx or suppression of G_{K-Ca} directly. The finding that spike potentials evoked in Na-free/high Ca solution and presumed to be generated by Ca currents were not significantly affected by d-Tc suggests that Ca influx across the membrane is not affected by d-Tc. Therefore, d-Tc appears to reduce spike AH by reducing G_{K-Ca} .

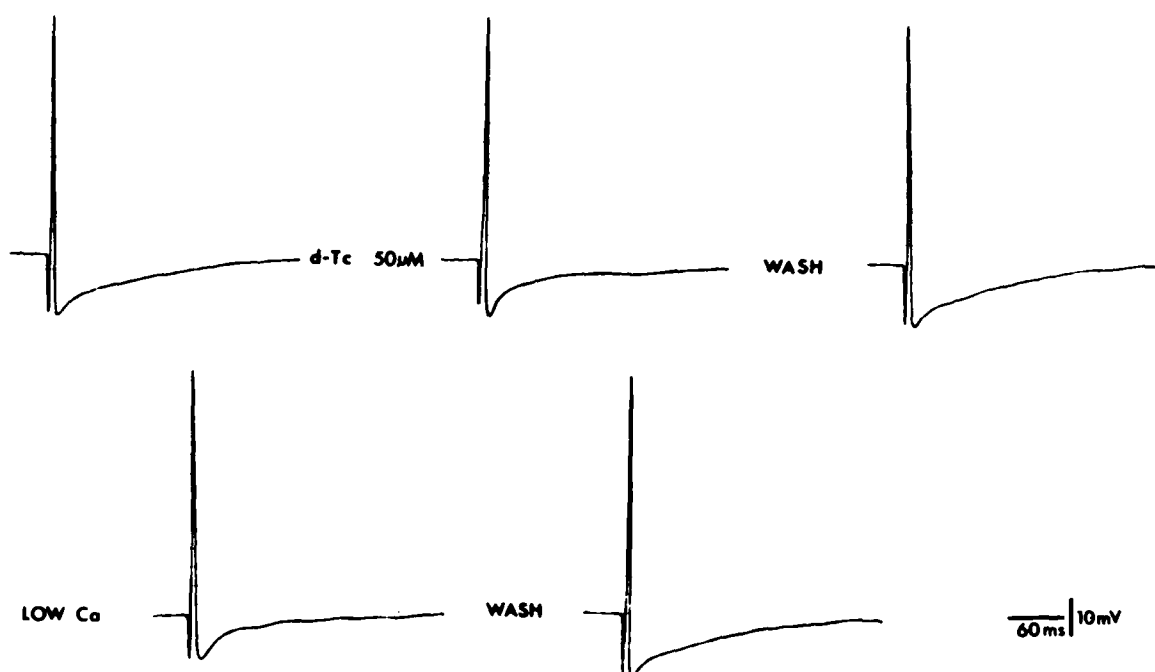


Fig. 12 Depression of spike AH by d-Tc in a rabbit superior cervical ganglion cell. Spike potentials were evoked by depolarizing current pulses injected through the recording microelectrode. Superfusion of d-Tc (50 μM) markedly shortened the duration of the spike AH. Low Ca (0.12 mM) solution similarly depressed the spike AH.

8. PGE_1 suppresses spike AH

PGE_1 is an endogenous autocoid that may be released from the ganglia during electrical stimulation (25). PGE_1 (10-500 nM) was found to reversibly depress 3 types of Ca-dependent potentials associated with the spike potential of rabbit superior cervical ganglion cells, namely, the spike AH, the post-tetanic hyperpolarization, and the Ca-spike evoked in a Na-free/high Ca solution (26). The results suggest that PGE_1 reduces Ca conductances and that this action may underlie its inhibitory action on ACh release from the ganglia (27).

IV. Discussion

The acute effects of OP agents including DFP on electrical responses of sympathetic neurons have not been investigated heretofore. The result obtained here demonstrates that DFP has a number of actions on synaptic transmission that are apparently unrelated to its well established ChE inhibition activity (10,28).

First, DFP exerted a dose-dependent facilitation and inhibition of nicotinic transmission in the rabbit superior cervical ganglia. The observation that DFP increased the nicotinic depolarizations induced by pressure ejection of ACh but not by CaCh indicates that the facilitatory action of DFP on nicotinic transmission is probably due to its ChE inhibition activity, resulting in a prolongation of the duration of action of ACh present at the synaptic junction (16). DFP, on the other hand, blocks nicotinic transmission at higher concentrations. The question that is of immediate interest is whether or not the blockade of ganglionic transmission by DFP was a result of accumulation of ACh, subsequent to inhibition of ganglionic ChE. Our results appear to indicate that DFP blocked ganglionic transmission by an action independent of ChE inhibition. The reasons are as follows. First, if the blockade of nicotinic transmission were a consequence of accumulation of ACh following inhibition of ChE by DFP, it would be expected that the blockade should be preceded by a depolarization accompanied by a reduction in membrane resistance (see also 14). However, DFP blocked nicotinic transmission without first causing a noticeable change of membrane potential or input resistance in 80% of the cells tested. Although DFP caused a small depolarization in a population of neurons, this action is apparently not involved in ganglionic blockade. Second and more importantly, the nicotinic depolarizations induced by pressure ejection of ACh and CaCh were effectively blocked by DFP suggesting that the latter acts postsynaptically. Although this finding does not necessarily exclude the possibility of a presynaptic site of action whereby DFP may alter transmitter release or block action potential propagation. Nevertheless, the fact that DFP blocks nicotinic depolarizations induced by exogenously applied ACh and CaCh is a strong indication that DFP abolishes nicotinic transmission by a postsynaptic site of action. The precise manner by which DFP blocks postsynaptic nicotinic response remains to be clarified. In this respect, we have shown recently that carbamate type of anti-ChE agents, physostigmine and neostigmine, also block nicotinic transmission by a postsynaptic mechanism (14).

The second interesting and novel finding relates to the effects of DFP on muscarinic transmission. Our result shows that DFP exerted a biphasic action on muscarinic transmission: it facilitates and depresses at low and high concentrations, respectively. The facilitatory effect of DFP at lower concentrations would be consistent with its ChE inhibition activity. However, the finding that muscarinic depolarizations induced by pressure ejection of MeCh, a muscarinic agonist that is slowly hydrolyzed by AChE, were also increased by lower concentrations of DFP suggests that the enhancement may not be a result of simple inhibition of ChE. The possibility that DFP at lower concentrations may have a direct muscarinic agonist activity remains to be further investigated.

In contrast to its facilitatory effect at lower concentrations, DFP at concentrations of 100 μ M or higher reversibly depressed muscarinic transmission. The inhibitory action appears to be a direct interaction of DFP with postsynaptic

muscarinic receptors/ion channels as the muscarinic depolarization elicited by pressure ejection of MeCh was similarly depressed.

In addition to its apparent direct muscarinic agonist and antagonist activity, DFP was found to alter muscarinic depolarization by converting it into a hyperpolarization in a portion of ganglion cells studied. Since the hyperpolarization was blocked by atropine, it appears safe to assume that the hyperpolarization induced by DFP was mediated by muscarinic receptors. The mechanism underlying this intriguing effect of DFP is not clear. Nevertheless, it is noteworthy that we have reported in an earlier study using surface recording techniques that carbamate type of anti-ChE agents, physostigmine and neostigmine, likewise converted muscarinic depolarization into a hyperpolarization (29). The question whether or not a similar mechanism may underlie this unusual effect of DFP and carbamate type of anti-ChE agents is interesting and remains to be addressed.

In summary, our results clearly show for the first time that DFP exerts an AChE-dependent facilitation and an AChE-independent inhibition of nicotinic and muscarinic transmission in the sympathetic ganglia. It is important in the context of OP toxicity that a rather narrow range of concentrations separates the enhancing effect of DFP from its inhibitory effect.

In the second half of studies, we demonstrate also for the first time that 2-PAM, a classic ChE reactivator, affects nicotinic transmission in a concentration dependent manner. The f-epsp's as well as nicotinic depolarizations induced by pressure ejection of ACh were increased and reduced by low and high concentrations of 2-PAM, respectively. The mechanism of the facilitatory action of 2-PAM on nicotinic transmission is not firmly established. It has been reported that 2-PAM exhibits mild anti-ChE activity (30). The observation that the nicotinic depolarizations induced by pressure ejection of ACh were increased by 2-PAM is consistent with the notion that the latter may enhance nicotinic transmission by an anti-ChE activity. Interestingly, 2-PAM inhibits nicotinic transmission at higher concentrations. This appears to be a direct action on nicotinic receptor/channel as the ACh depolarizations induced by pressure ejection of ACh was similarly blocked. Thus, our result seems to suggest that 2-PAM may have a d-Tc like action, albeit at a high concentration.

Two minor but related studies were carried out during the past year, namely the effects of d-Tc and PGE_1 on spike AH. We found that d-Tc and PGE_1 both depress spike AH by apparently two different mechanisms. PGE_1 reduced G_{Ca} , whereas d-Tc inhibits $G_{\text{K-Ca}}$. What is the consequence of reduction of spike AH in terms of neuronal function? The frequency and pattern of spike discharge of a neuron is controlled by the duration of spike AH a reduction of which may lead to convulsive phenomena in the vertebrate neurons (31). It is interesting in this respect that d-Tc when applied to cortical neurons causes convulsive activities (31). The implication is that by shortening the spike AH d-Tc may enhance repetitive firings in sympathetic neurons. In the case of PGE_1 , a reduction of G_{Ca} in the nerve terminal membrane may cause a reduction of transmitter release. This may explain the depressant effect of PGE_1 on ACh release in the ganglia (27).

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VI. Publications:

- Mo, N. and Dun, N. J. (1984). Vasoactive intestinal polypeptide facilitates muscarinic transmission in mammalian sympathetic ganglia. Neurosci. Letts. 52, 19-23.
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Presentation:

- Mo, N., Jiang, Z. G., Dun, N. J. and Karczmar, A. G. (1985). D-tubocurarine suppresses spike after-hyperpolarization in guinea pig prevertebral neurons. Annual Society for Neuroscience Meeting.

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
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4. TITLE (and Subtitle) CELLULAR ACTIONS AND INTERACTIONS OF ANTICHOLIN- ESTERASES AND THEIR ANTIDOTES IN MAMMALIAN AUTO- NOMIC NEURONS		5. TYPE OF REPORT & PERIOD COVERED Annual Summary Report 15 April 1984-14 April 1985
7. AUTHOR(s) N.J. Dun, Ph.D.		6. PERFORMING ORG. REPORT NUMBER
9. PERFORMING ORGANIZATION NAME AND ADDRESS Loyola University Stritch School of Medicine 2160 S. First Avenue Maywood, IL 60153		8. CONTRACT OR GRANT NUMBER(s) DAMD17-83-C-3133
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701-5012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62734A3M162734A875.AA.437.
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE July 29, 1985
		13. NUMBER OF PAGES 28
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Organophosphorous anti-cholinesterase agents, diisopropylfluorophosphate, sympathetic ganglia, pyridinealdoxime, fast excitatory postsynaptic potential, slow excitatory postsynaptic potential, nicotinic transmission, muscarinic transmission, d-tubocurarine, prostaglandin E ₁ , spike afterhyperpolarization, acetylcholinesterase, cholinesterase.		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The effects of an organophosphorus anti-cholinesterase (anti-ChE) agent, diisopropylfluorophosphate (DFP) and pyridinealdoxime (2-PAM), an acetylcholinesterase (AChE) reactivator, on neurons of the isolated rabbit superior cervical ganglia and on ganglionic transmission were investigated by means of intracellular recording techniques. DFP exerted a dose-dependent action on nicotinic and muscarinic transmission of the sympathetic neurons. At concentrations of 10 µM or less, DFP increased the amplitude as well as the duration of the fast		

excitatory postsynaptic potential (f-epsp) which is nicotinic in nature. The nicotinic acetylcholine (ACh) depolarization induced by ACh applied by pressure ejection was likewise increased. On the other hand, DFP at concentrations of 0.1 mM or higher consistently and reversibly depressed f-epsp's and ACh depolarizations. The effects of DFP on depolarizations induced by carbamylcholine (CaCh), a ChE resistant cholinergic agonist, were concentration dependent. DFP at concentrations of 10 μ M or less did not noticeably change the amplitude or duration of CaCh-induced depolarizations, whereas, the latter were reversibly abolished by DFP at concentrations of 0.1 mM or higher. The effects of DFP on muscarinic transmission were also concentrations dependent. DFP at concentrations of 10 μ M or less increased the amplitude and duration of the slow excitatory postsynaptic potential (s-epsp) which is muscarinic in nature as well as the depolarization induced by methacholine (MeCh), a specific muscarinic agonist that is slowly hydrolyzed by AChE. On the other hand, DFP at concentrations of 0.1 mM or higher reversibly blocked the s-epsp as well as MeCh-induced depolarization. 2-PAM increased and decreased the nicotinic depolarizations whether evoked synaptically or by pressure ejection of ACh at concentrations of 1 mM or lower and 10 mM or higher, respectively. Thus, the results provide evidence for the first time that DFP and 2-PAM exerted an AChE dependent facilitation and an AChE independent inhibition of nicotinic transmission. It is therefore concluded that contrary to the long standing concept that DFP and 2-PAM affect cholinergic transmission solely by inhibiting and reactivating junctional AChE, respectively, these compounds exert direct actions on cholinergic nicotinic and muscarinic receptors/ion-channels complex that are independent of the status of junctional AChE activity. In addition to these two major studies, it was found that d-tubocurarine (d-Tc, 10-100 μ M) and prostaglandin E_1 (PGE₁, 10-100 nM) reversibly and dose-dependently suppressed the spike after-hyperpolarization (AH) of rabbit and guinea pig sympathetic ganglion cells. The ability of these two compounds to inhibit spike AH is interesting as they may increase the membrane excitability of sympathetic neurons and other central neurons.

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