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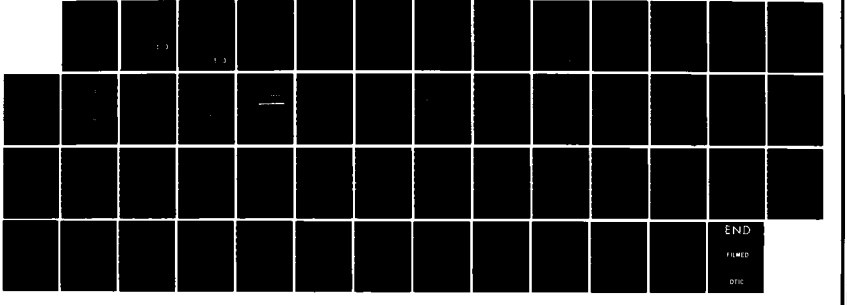

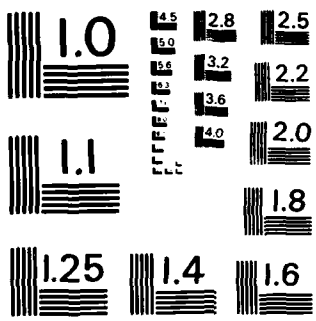

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Annual Summary Report

Frank J. Lebeda, Ph.D.
Paul A. Rutecki, M.D.
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October 1983

Supported by

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

Contract No. DAMD17-82-C-2254

Department of Neurology
Baylor College of Medicine
Houston, Texas 77030

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER	2. GOVT ACCESSION NO	3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle) SYNAPTIC MECHANISMS OF ACTION OF CONVULSION-PRODUCING ANTICHOLINESTERASES: CHARACTERIZATION OF DI-ISOPROPYL PHOSPHOROFLUORIDATE-INDUCED EPILEPTIFORM ACITIVITY IN THE MAMMALIAN HIPPOCAMPUS.

5. TYPE OF REPORT & PERIOD COVERED
Annual Summary
September 1982-September 1983

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
Frank J. Lebeda, Paul A. Rutecki, and Daniel Johnston

8. CONTRACT OR GRANT NUMBER(s)
DAMD17-82-C-2254

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Department of Neurology
Baylor College of Medicine
1200 Moursund, Houston, TX 77030

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
6.27.34.A
3M162734A875
AA.380

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD 21701

12. REPORT DATE
October 1983

13. NUMBER OF PAGES
48

14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*

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)*

Epilepsy
di-isopropyl phosphorofluoridate
Hippocampus
4-aminopyridine
tetraethylammonium

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The purpose of this work was to elucidate the convulsant mechanisms of action of the irreversible organophosphorus anticholinesterase (anti-ChE) di-isopropyl phosphorofluoridate (DFP). In these experiments pyramidal cell responses from rat and guinea-pig hippocampal slices were examined in vitro using standard extra- and intracellular microelectrode recording techniques.

When bath-applied at 10-25 μM for 30 min, DFP produced spontaneous, rhythmic discharges (50-400 msec in duration at 0.1-0.3 Hz) in the pre-

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viously quiescent extracellular field recordings. This activity was superficially similar to that produced by other convulsants, e.g., bicuculline and picrotoxin, which interfere with inhibitory synaptic activity that is mediated by gamma-aminobutyric acid.

This DFP-induced effect was readily abolished upon washout, even in slices that were exposed for up to 5 hrs. Reintroduction of the DFP solution reinitiated these epileptiform events. The DFP-induced events, however, were not suppressed with the cholinesterase reactivator pralidoxime (10-100 μM), but were abolished by high concentrations of atropine (10-100 μM).

Other anti-ChEs tested were the carbamates neostigmine, physostigmine and pyridostigmine (10-500 μM). None of these agents was observed to produce epileptiform activity within the 30 min test period. Similarly, no abnormal field activity was observed with bath-applied acetylcholine (2 mM) or carbachol (1 mM). In contrast, 10 μM of the organophosphate ethylbicyclophosphorothionate (EBPT), which does not have anti-ChE activity, induced pronounced epileptiform discharges.

At higher concentrations (25-50 μM) DFP produced relatively long-lasting discharges (ca. 1000-3000 msec) in the extracellular records. At the highest concentrations examined (50-60 μM), DFP produced extraordinarily large (10-20 mV) negative DC shifts in potential that were 1-2 min in duration and occurred approximately every 5 min, a phenomenon that may represent the in vitro correlate of the spreading cortical depression of Leao. Similar shifts were seen in vitro with high extracellular potassium concentrations (above 10 mM), but only rarely with convulsants that block inhibitory neurotransmission in the hippocampus.

Intracellular studies that examined the brief, periodic discharges initiated by 10 μM DFP clearly demonstrated that an excitatory postsynaptic potential underlies the drug-induced event. The apparent reversal potential of this DFP-induced paroxysmal depolarizing shift (PDS) was about 25 mV more negative than that of a pure excitatory event, suggesting that a substantial inhibitory synaptic component was still present.

Unlike the better characterized convulsants, DFP did not abolish inhibitory synaptic transmission. Inhibitory postsynaptic potentials (IPSPs) occurred spontaneously between the DFP-induced PDSs in marked contrast to what is observed with other convulsants.

Agents that reduce potassium-mediated currents in hippocampal neurons were also examined. Both tetraethylammonium (TEA; 1-10 mM) and 4-aminopyridine (4AP; 1-50 μM) produced a spectrum of epileptiform behavior that was strikingly similar to that induced by DFP. In addition, neither of these agents abolished inhibitory synaptic activity.

These results support the idea that the mechanism by which DFP induces PDSs in this central nervous system preparation is not directly related to its well-known anti-ChE activity. Furthermore, the present data are inconsistent with the hypothesis that a reduction of inhibitory synaptic transmission is the primary epileptogenic mechanism for DFP. The present working hypothesis is that DFP produces epileptiform activity, at least in part, by acting in a manner similar to 4AP and TEA, ie., blocking one or more of the several species of outward (potassium) currents that have been described in these neurons. The possibility that one of these potassium currents is gated by acetylcholine is also discussed.

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators 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 Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

I. INTRODUCTION

A large number of centrally acting drugs with diverse chemical structures can produce convulsant activity. Within this group of convulsants are the organophosphorus (OP) anticholinesterases (anti-ChE) [1,2,3,4], which are thought to act primarily by raising the level of acetylcholine (ACh) at central synapses, thereby accentuating the known excitatory effects of this neurotransmitter [5,6,7,8].

In the course of studying the convulsant mechanism of d- ubocurarine on the in vitro hippocampal slice preparation [9], it became evident that this classic, peripheral nicotinic blocker produced epileptiform activity in a manner similar to that of better characterized convulsants, e.g., bicuculline and picrotoxin. The latter agents interfere with inhibitory synaptic activity mediated by gamma-aminobutyric acid (GABA), perhaps the major inhibitory neurotransmitter in the supraspinal regions of the central nervous system (CNS) [10,11]. Additional results indicated that other cholinergic blockers (e.g., alpha-bungarotoxin, atropine) did not produce abnormal neuronal discharges. In light of this disparity between the central and peripheral mechanisms of action of d-tubocurarine, the question arose as to the mechanism of action for the well-established epi' 'form activity that can be induced by anti-ChE agents.

A systematic approach was taken in this study to examine the convulsion-producing effects of OP anti-ChEs at the cellular leve 'n the CNS. In pursuing the long-term goal of determining how this adverse, drug-induced reaction can be prevented or alleviated, we have chosen di-isopropyl phosphorofluoridate (DFP) as the initial test agent. One set of experiments described in this report dealt with elucidating the mechanism of the seizure-inducing action of this drug. In future studies, using similar electrophysiological techniques, we will examine a variety of clinically used anticonvulsants whose cellular mechanisms are only partially known at this time. By understanding how these drugs can produce or prevent abnormal neuronal activity within single neurons and within populations of neurons that compose the richly interconnected networks of the CNS, it may be possible to combat drug-induced convulsant effects with drugs having diametrically opposed molecular mechanisms. A similar, methodical approach has been taken in the ongoing development and study of the oxime group of ChE reactivators [3,12,13,14,15,16].

In this pharmacological study of the convulsant effects of DFP, we have used the in vitro hippocampal slice preparation [17], which has several advantages. Perhaps foremost, the hippocampus is generally regarded to be one of the most susceptible regions of the CNS to convulsion-producing drugs. This preparation also serves as a valuable screening tool for examining drug action upon mammalian CNS responses, i.e.; drugs at known concentrations can be bath-applied directly onto the slices without having the blood-brain barrier diffusion problems that are encountered in vivo. Finally, because of the compact, laminar cytoarchitecture of the hippocampus, extracellular field recordings from populations of cells, particularly the large, pyramidal neurons in the CA1 and CA3 regions, can be studied. These cell body layers also serve as valuable target areas for positioning microelectrodes for intracellular

recordings. This anatomical organization thus makes it technically feasible to correlate population with intracellularly recorded electrical activity.

In characterizing the mechanism causing DFP's convulsant effects, two hypotheses were considered. The first was that the classic, anti-ChE action of this drug is responsible for the observed epileptiform activity. Several predictions of this hypothesis were derived and tested: 1) The convulsant action of this agent should be irreversible; 2) similar convulsant actions should be induced by cholinergic agonists and by other classes of anti-ChEs; 3) these DFP-induced effects should be blocked by ChE reactivators and by muscarinic antagonists.

The second hypothesis considered was that DFP interferes with inhibitory neurotransmission. This hypothesis was formulated on the basis of the action of several other convulsants that are known to block GABA-mediated inhibitory synaptic activity. It seemed reasonable to suspect that the convulsant action of DFP might be similar to that produced by these other agents. Several predictions stemming from this hypothesis were also derived and tested: 1) Inhibitory synaptic events should be severely reduced or abolished by DFP; 2) the waveform of the DFP-induced event recorded intracellularly under current-clamp conditions should clearly reverse in polarity at or near 0 mV [9,18], a result which would indicate that an excitatory postsynaptic potential (EPSP) is responsible for producing these epileptiform discharges.

As described in the following sections, our experimental findings were inconsistent with the predictions derived from each hypothesis. Another hypothesis was therefore formulated, based upon the observation that epileptiform activity similar to that produced by DFP can be generated by the potassium channel blockers tetraethylammonium (TEA) [19,20] and 4-aminopyridine (4AP) [21]. Since it has recently been suggested [5,6,8,22] that ACh mediates excitatory activity in the hippocampus by reducing a steady potassium current, it is possible that DFP is interacting directly with these ACh-gated potassium channels (in addition to the other potassium channels) in producing epileptiform events. Further elaboration of the latter hypothesis is presented in the Discussion (Section IV.E.). Preliminary descriptions of this work have appeared as abstracts [23,24].

II. MATERIALS AND METHODS

A. Animals and Slice Preparation

The methods of preparing hippocampal slices are similar to those described in previous studies [9,25,26]. In preliminary experiments (see Figure 5), pigmented guinea pigs of either sex, weighing 200-400 g, were used. Later experiments were conducted with Sprague-Dawley male rats (100-200 g). The experimental data from these animals were used in the remaining figures. Following decapitation, the brains were quickly removed and placed on filter paper moistened with chilled saline solution, and the hippocampi were dissected free. Hippocampi were sliced with a MacIlwain tissue chopper transverse to the longitudinal axis and were nominally

400-600 microns thick. With a fine brush, 6 to 10 slices were transported to the humidified recording chamber (modified from the design of Haas et al. [27]), where they were continuously superfused with an oxygenated (95% O2, 5% CO2) physiological saline at 32-35~ C.

B. Stimulation and Recording Techniques

Glass microelectrodes were used for recording both extra- and intracellular responses from the CA3 pyramidal subfields (see Figure 1, [28]). For intracellular microelectrodes, the capillary tubes were filled, then pulled with strands of fiber-glass to expedite subsequent filling with either 4 M potassium acetate or 2 M cesium sulfate [25]. Resultant tip resistances ranged from 20 to 80 Mohms. Loading individual neurons with cesium resulted in reduced potassium-dependent outward currents, and relatively small injection currents (<5 nA) were required to change the membrane potential within the range of about -120 to +30 mV. Extracellular electrodes were filled only with potassium acetate.

Teflon-coated bipolar stimulating electrodes were used to evoke orthodromic, synaptic responses. The CA3 population of neurons was orthodromically activated by placing the metal electrode in the hilus of the area dentata (AD) and stimulating the mossy fibers (MF) area. Orthodromic activation of the CA1 subfield was achieved by stimulating the stratum radiatum to activate the Schaffer collaterals (SC). Both stimulating and recording electrodes were positioned with hydraulic manipulators under visual control, using a low magnification dissecting microscope.

In some studies a conventional bridge amplifier circuit was used to monitor the neuronal responses. In most of the experiments a switched (3 kHz) sample-and-hold circuit [25,26,29] was used for current-clamping with a single microelectrode. This circuit obviates bridge amplifier balancing problems that can introduce significant errors in the measurement of the membrane potential when current is injected. Rectangular pulses from a WPI stimulator and neuronal responses were observed on a storage oscilloscope. These signals were collected on both a Gould/Brush paper chart recorder and a Racal FM tape recorder (frequency response: DC to 5 kHz).

C. Data Analysis

Data stored on FM tapes were digitized and subsequently analyzed using a custom-configured laboratory computer based on a DEC LSI-11/23 system with 256 Kbytes RAM. A specialized interface unit and software package (BASIC-23/HP) were obtained from Cheshire Data and were used in conjunction with a Data Translation A-to-D converter (maximum single channel rate: 125 kHz). Under software control, a Schmidt trigger was used to initiate sampling at 1-2 kHz from 1 or 2 A-to-D channels. Spontaneously occurring synaptic potentials were sampled in a continuous mode at the same rate of digitization. Digitized waveforms were stored in binary files on a Winchester fixed disk (15 Mbytes) and later backed up on flexible disks. Waveforms could then be recalled from disk and either displayed on an Hewlett-Packard HP 1345A vector monitor or plotted on an HP 7470A digital XY plotter. When displayed on the monitor, these waveforms were analyzed

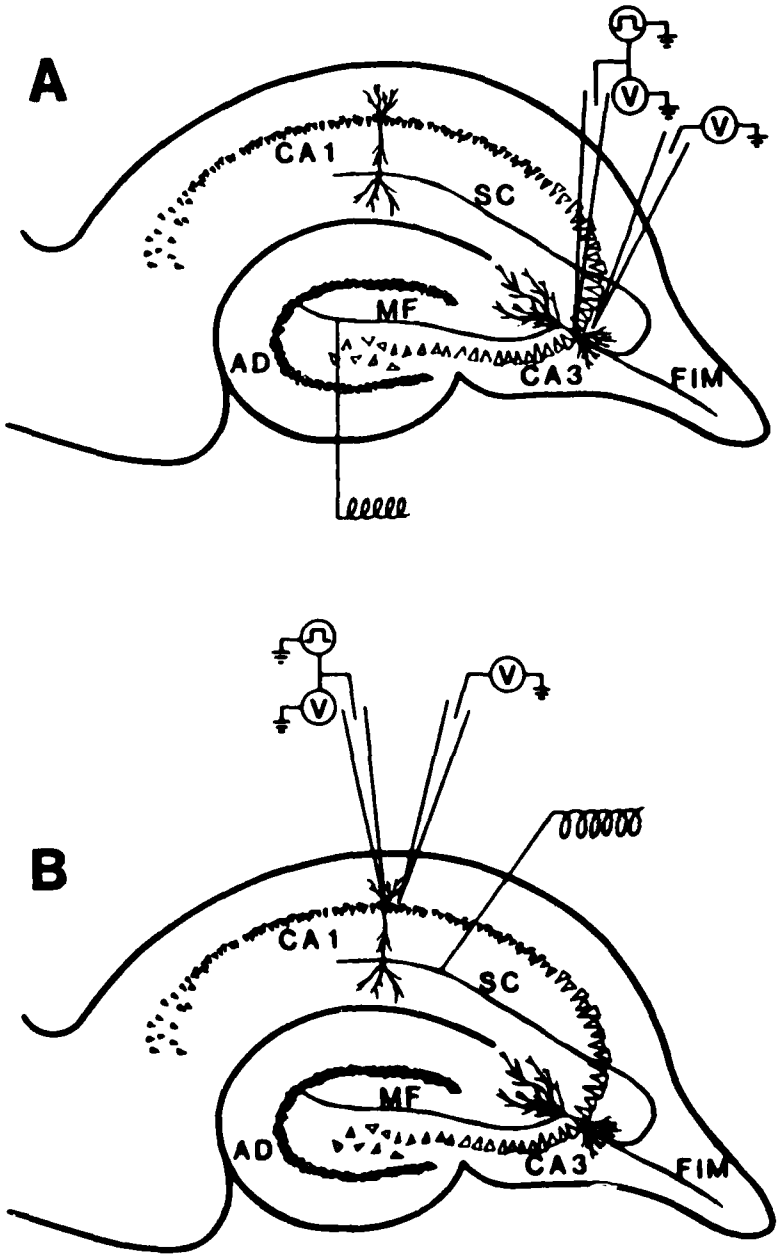


FIGURE 1.

Schematic diagrams of the hippocampal slice showing placement of stimulating and recording electrodes. A metal stimulating electrode was placed in the area of the mossy fibers (MF) in the hilus of the area dentata (AD) or in the stratum radiatum of the CA3 area to activate the Schaffer collaterals (SC). Extra- and intracellular glass microelectrodes were positioned in the pyramidal cell body layers of the CA3 or CA1 region.

with a software package that used either an automated routine or a set of user-interactive cursors. Both network modeling and statistical analysis for calculating means, standard errors of the mean, and least squares linear regression were done, using this computer system.

D. Solutions and Drugs

The physiological (control) saline had the following composition (in mM): NaCl, 124; KCl, 5; NaH_2PO_4, 1.25; $NaHCO_3$, 26; $CaCl_2$, 2; $MgSO_4$, 2; glucose, 10. For experiments in which the effects of various potassium concentrations were studied (2.5-12 mM), no adjustments were made to correct for the small change in the osmolarity of the resultant solution.

All of the DFP used in this study was obtained as a single shipment from Calbiochem (La Jolla, CA; lot no. 103288) in order to reduce the amount of variability in potency that has been reported with commercially supplied samples [30]. Dilute stock solutions of DFP were typically made immediately before being added to the saline reservoir. The unused dilute stock was then frozen for later experiments.

Pyridostigmine bromide was a gift from Hoffmann-La Roche (Nutley, NJ) and bicuculline methiodide was purchased from Pierce Chemical Co. (Rockford, IL). Dr. Eugene M. Barnes kindly provided the ethylbicyclophosphorothionate (EBPT). The other drugs used were purchased from Sigma (St. Louis, MO): acetylcholine chloride, 4-aminopyridine, atropine sulfate, carbamylcholine chloride (carbachol), neostigmine bromide, pyridine-2-aldoxime methiodide (2-PAM), physostigmine sulfate (eserine sulfate), picrotoxin and tetraethylammonium chloride. The molecular structures of the convulsants used are depicted in Figure 2.

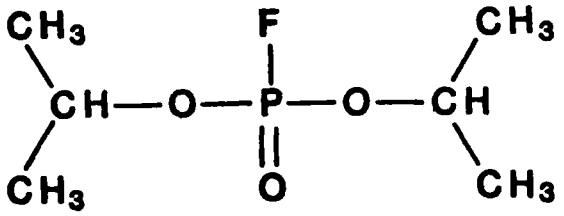
For screening experiments, solutions containing various amounts of candidate drugs were bath-applied onto the slices for a test period of 30 min. During this time, extracellular recording and stimulating electrodes were in place to determine whether the agent being examined could induce spontaneous or orthodromically evoked repetitive field discharges.

III. RESULTS

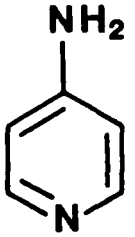
A. Types of Epileptiform Activity Induced by DFP

Under control conditions, extracellular recordings from CA3 subfields of either rat or guinea pig hippocampal slices were typically inactive, with only an occasional spontaneous unit discharge being observed. Within 15 min of bath-applying 10-25 μM DFP, a gradual emergence of single unit activity took place. Since this activity seemed to be more vigorous in the rat hippocampus ($n = 9$) than in the guinea pig preparation ($n = 3$), rat hippocampi are used in subsequent experiments with DFP.

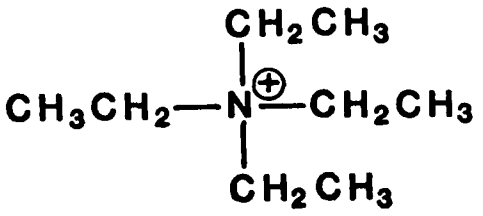
After an additional 15 min exposure, DFP produced spontaneously occurring, repetitive discharges resembling those events induced by other previously examined convulsants (e.g., picrotoxin, penicillin, bicuculline) and the other convulsants used in this study (e.g., 4AP, TEA and solutions containing high concentrations of potassium) (Figure 3). These DFP-induced



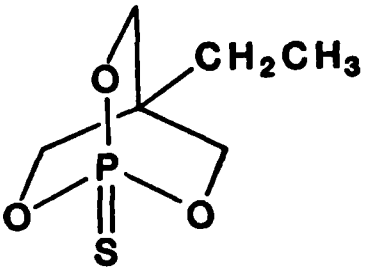
DFP



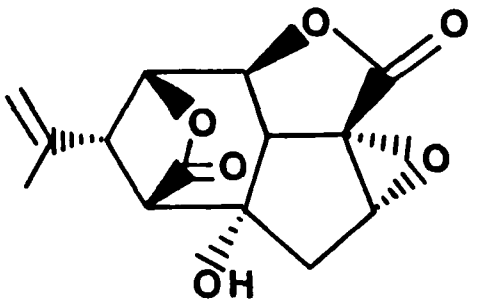
4AP



TEA



EBPS



PICROTOXININ

FIGURE 2.
Molecular structures of the convulsants used in this study. Diisopropyl phosphorofluoridate (DFP), 4-aminopyridine (4AP), tetraethylammonium (TEA), picrotoxinin (the active component of picrotoxin) ethylbicyclophosphorothionate (EBPT).

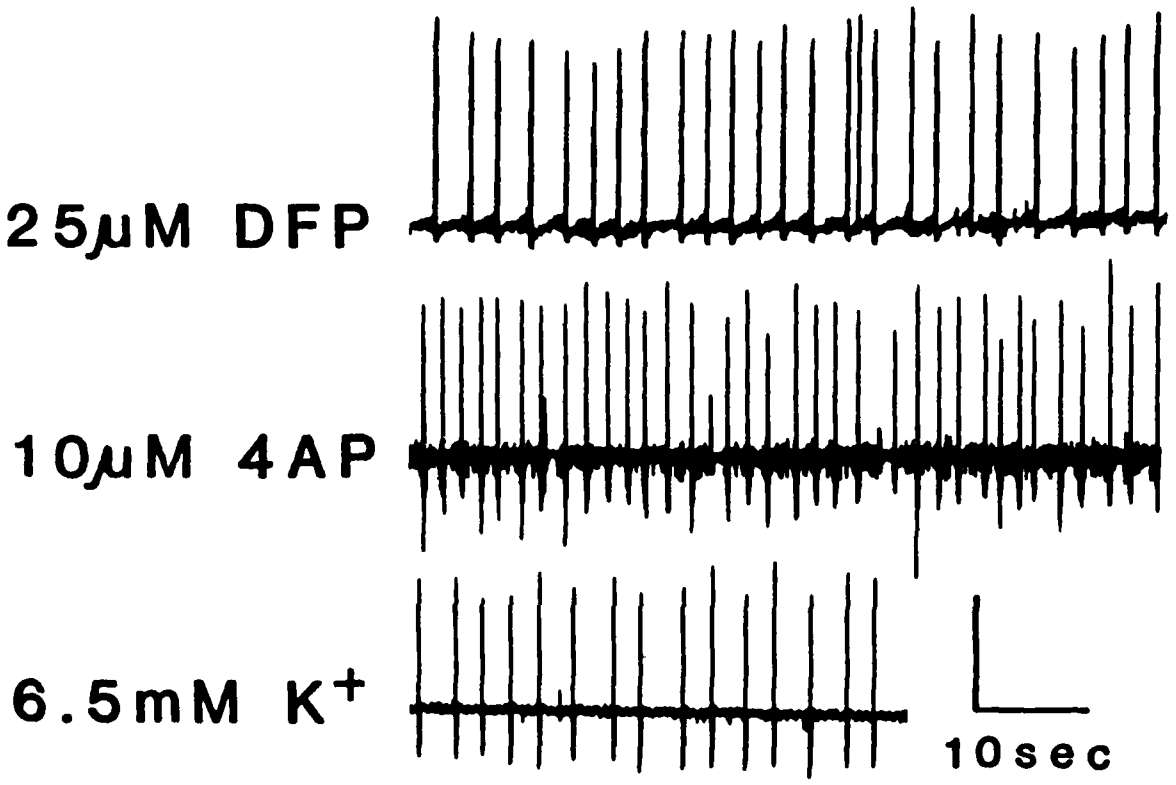


FIGURE 3.

Periodic discharges induced by various convulsants and high extracellular potassium at approximately equipotent concentrations. These extracellular recordings were obtained in the CA3 region of different hippocampal slices. The discharges were 50-300 msec in duration and occurred at 3-5 sec intervals. The field polarities referred to in this work conform to the intracellular convention; i.e. negative is a downward deflection in the traces shown. Voltage calibration is 1 mV for the top two traces and 0.5 mV for the bottom trace.

field discharges occurred every 3-10 sec and lasted 50-400 msec. A silent interval lasting 100-200 msec usually followed each field discharge.

Such extracellularly recorded drug-induced discharges have been considered by a number of investigators to be analogous to the interictal spikes seen in the EEG recordings from epileptic patients. By definition, these discharges are not seizures. Rather, these spontaneously occurring discharges reflect a preseizure condition [31,32] and represent a rudimentary form of epileptic behavior. These waveforms are stereotypic in appearance and are regularly occurring events, with explosive onsets and rapid terminations that arise from synchronous neuronal discharges. The mechanisms responsible for the transition between the interictal to a full-blown seizure state are presently unclear, but knowing the cellular basis of these periodic events is expected to help in our understanding of how seizure activity arises and spreads within the CNS.

As illustrated in Figure 4, the extracellularly recorded event was accompanied by an intracellularly recorded discharge comprising an envelope of depolarization, upon which were superimposed a number of action potentials. This envelope of depolarization was originally termed the paroxysmal depolarizing shift (PDS) by Matsumoto and Ajmone-Marson [31], in characterizing penicillin-induced discharges. Both the population responses and the intracellular PDSs were quite stereotypic in appearance and usually had the waveforms shown in panel A of Figure 4. Less frequently, either the intracellular event outlasted the extracellular response (panel B) or the opposite pattern occurred (panel C). Panel D shows prolonged extra- and intracellular responses. Thus, in general, the intracellular recordings reflected the stereotypic behavior noted in the previous extracellular observations.

The range of effects at different DFP concentrations is summarized in Figure 5. Panel A illustrates the previously noted synchronous, periodic activity in the field records with 25 μM DFP. At a higher concentration, DFP induced a secondary discharge, lasting about 3 sec (Panel B). This longer event was followed by a relatively inactive period and, later, by the return of the brief discharges. This sequence of events had its own periodicity, occurring approximately once every 30 sec.

At the highest concentrations of DFP tested so far (50-60 μM; n = 3), these periodically occurring discharges were replaced by exceptionally large, negative DC shifts in potential (Figure 5C1, C2). In this preparation, 50 μM DFP caused 10-20 mV shifts that were 1-2 min in duration and occurred every 5 min. During these shifts, orthodromic stimulation could not evoke any detectable response. Between these spontaneous shifts, however, responses could be evoked and on occasion produced an additional, large DC shift. In Panels C2 and C3, a washout was attempted. Even though these large field potentials seemed to be abolished upon washout, this apparent reversible effect of DFP requires further evaluation.

B. Synaptic Nature of the DFP-induced PDS

To investigate further the DFP-induced PDSs, we used relatively low concentrations (10-25 μM) of DFP and intracellular recordings. In these

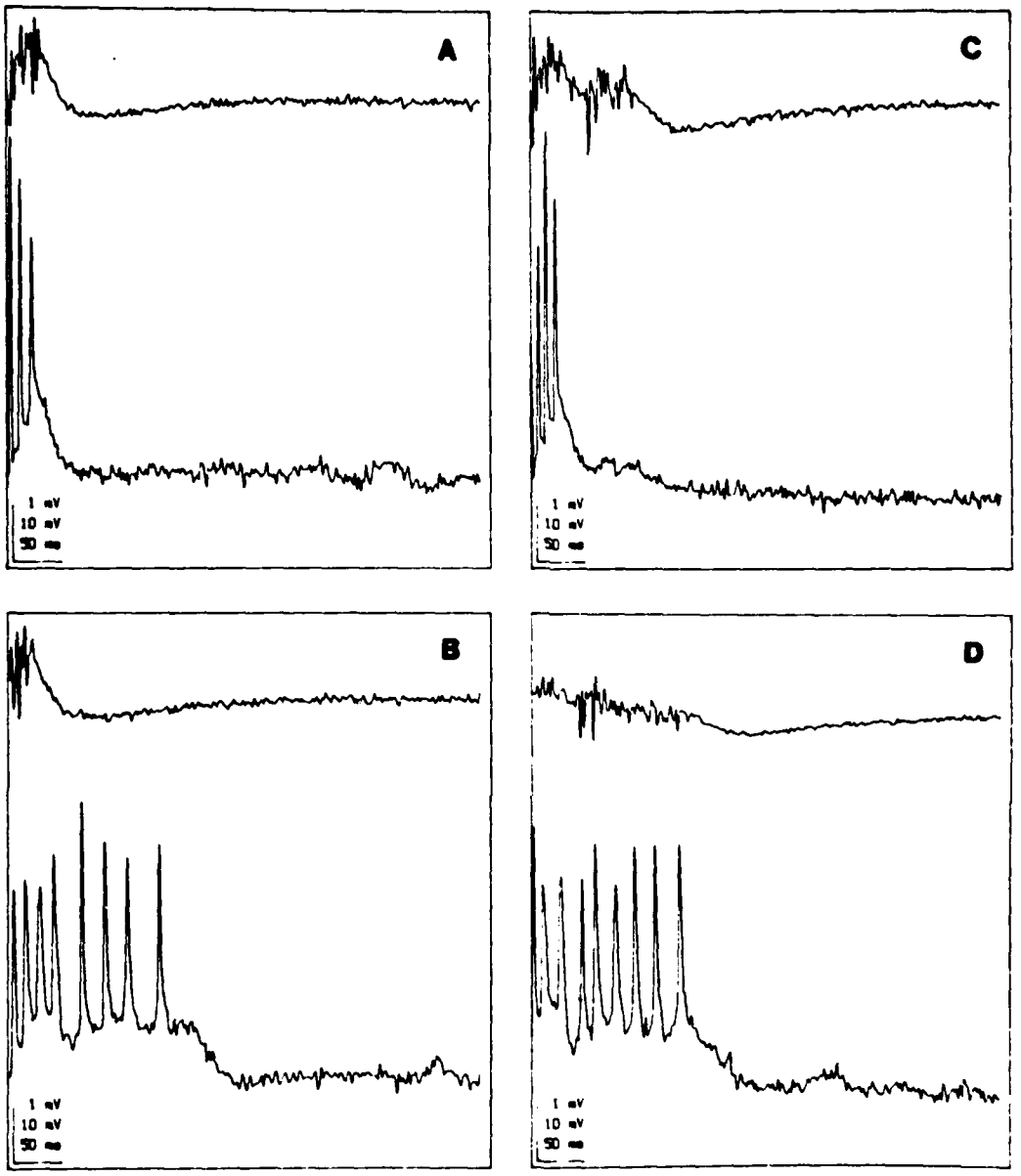


FIGURE 4.

Examples of discharge patterns induced by DFP recorded concurrently with extra- and intracellular electrodes. Panel A: Typical intracellularly recorded (top trace) spontaneous PDS (paroxysmal depolarizing shift) accompanied by an extracellularly recorded discharge (bottom trace) of a similar duration. More than 90% of these events had this waveform. Panels B and C: Occasionally either the intracellularly or the extracellularly recorded waveform was more prolonged than the companion recording. Panel D: In some cases both recorded events were equally prolonged. Recordings taken from two cells exposed to 25 μM DFP. Panels A,B,C: Resting membrane potential (RMP) = 65 mV. Panel D: RMP = 60 mV.

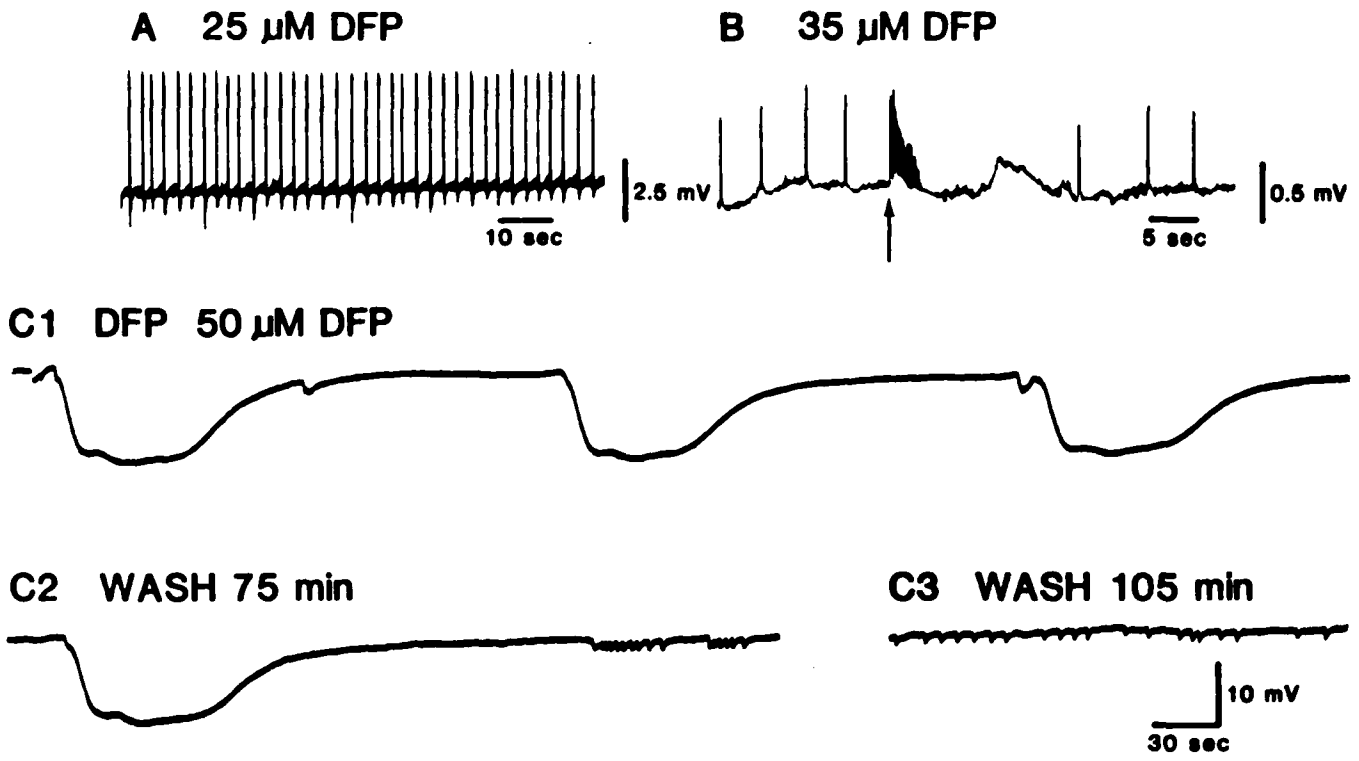


FIGURE 5.

Spectrum of DFP-induced epileptiform activity seen in the hippocampal slice using extracellular recording techniques. Panel A: Periodic, synchronous discharges induced with 25 µM DFP. Panel B: 35 µM DFP induced a prolonged discharge (ca. 5 sec) of repetitive activity followed by a relatively quiescent period. This abnormal pattern was superimposed on the slower discharge frequency seen in Panel A. This longer discharge recurred every 30 sec in this preparation. Panel C1: Another preparation showing spontaneously occurring DC shifts, which were 1-2 min in duration and appeared every 5 min. The polarity has been maintained, but note the compressed voltage calibration. During these events, evoked, orthodromic responses could not be elicited. Panels C2 and C3: Same preparation during washout of DFP. Spontaneous events were still present but the DC shifts were abolished.

experiments, the objective was to determine whether the depolarizing shift was due to voltage-dependent processes or to a synaptic event. A more extensive description of these two alternative mechanisms and their distinguishing characteristics will be presented in the Discussion (Section IV.A.; see also [9,18]). The results in Figure 6 illustrate that a synaptic mechanism can readily account for these DFP-induced changes. At -64 mV, the spontaneously occurring PDS was either a subthreshold depolarization or a depolarizing shift which attained threshold and triggered a series of superimposed action potentials. With a small amount of current, the membrane was depolarized to -48 mV and was associated with a smaller, spontaneous shift. At <0 and +16 mV the initial part of the waveform was reversed in polarity. When the amplitudes of these paroxysmal events were plotted as a function of membrane potential, as in Figure 7, the relation was monotonic with an apparent reversal potential of -26 mV. This interpolated value is near the reversal potential of a mixed synaptic event comprising both excitatory and inhibitory synaptic inputs [33].

The voltage-independence of the DFP-induced PDS is illustrated in Figure 8 and provides further evidence of the synaptic nature of this abnormal event. Over a range of 100 mV, the PDS frequency remained near 0.2 Hz. For comparative purposes the endogenous burst frequency from a CA3 neuron under control conditions is also shown. The relationship is highly nonlinear over the relevant range of membrane potentials.

C. The Anti-ChE Hypothesis for the DFP-induced PDS

As outlined in the Introduction, the first hypothesis to be tested to account for the production of epileptiform activity by DFP involves its well-known, irreversible blockade of ChE activity. The first prediction of this hypothesis was that the action of DFP should be irreversible. As illustrated in Figure 9, the periodic discharges induced by 25 μM DFP were readily eliminated upon washout. In this experiment, the preparation was exposed to DFP for 5 hours before washout. Cessation of the spontaneous discharges occurred within 10 min. To ensure that the preparation had not deteriorated or that the DFP in the solution had not undergone spontaneous hydrolysis, the DFP-containing saline solution from the original reservoir was reintroduced. Within 30 min, the same type of discharge pattern was observed in the same slice.

The second prediction of the anti-ChE hypothesis for DFP stated that similar epileptiform effects should be produced by cholinergic agonists and other anticholinesterase agents. Neither acetylcholine (2 mM) nor carbachol (1 mM) (Table 1) was able to produce spontaneous or evoked repetitive field activity within the 30 min test period. In addition, no depressant effect on the control population response was noted. Similarly, none of the carbamate anticholinesterases examined (neostigmine, 100-1000 μM; pyridostigmine, 100-200 μM; physostigmine, 10-500 μM) could induce epileptiform discharges during this testing period.

The third prediction of the anti-ChE hypothesis for DFP's epileptogenic action was that ChE reactivators and muscarinic antagonists should counteract the discharges induced by DFP. In these experiments, the preparations were initially exposed to 25 μM DFP (30-60 min; Table 1).

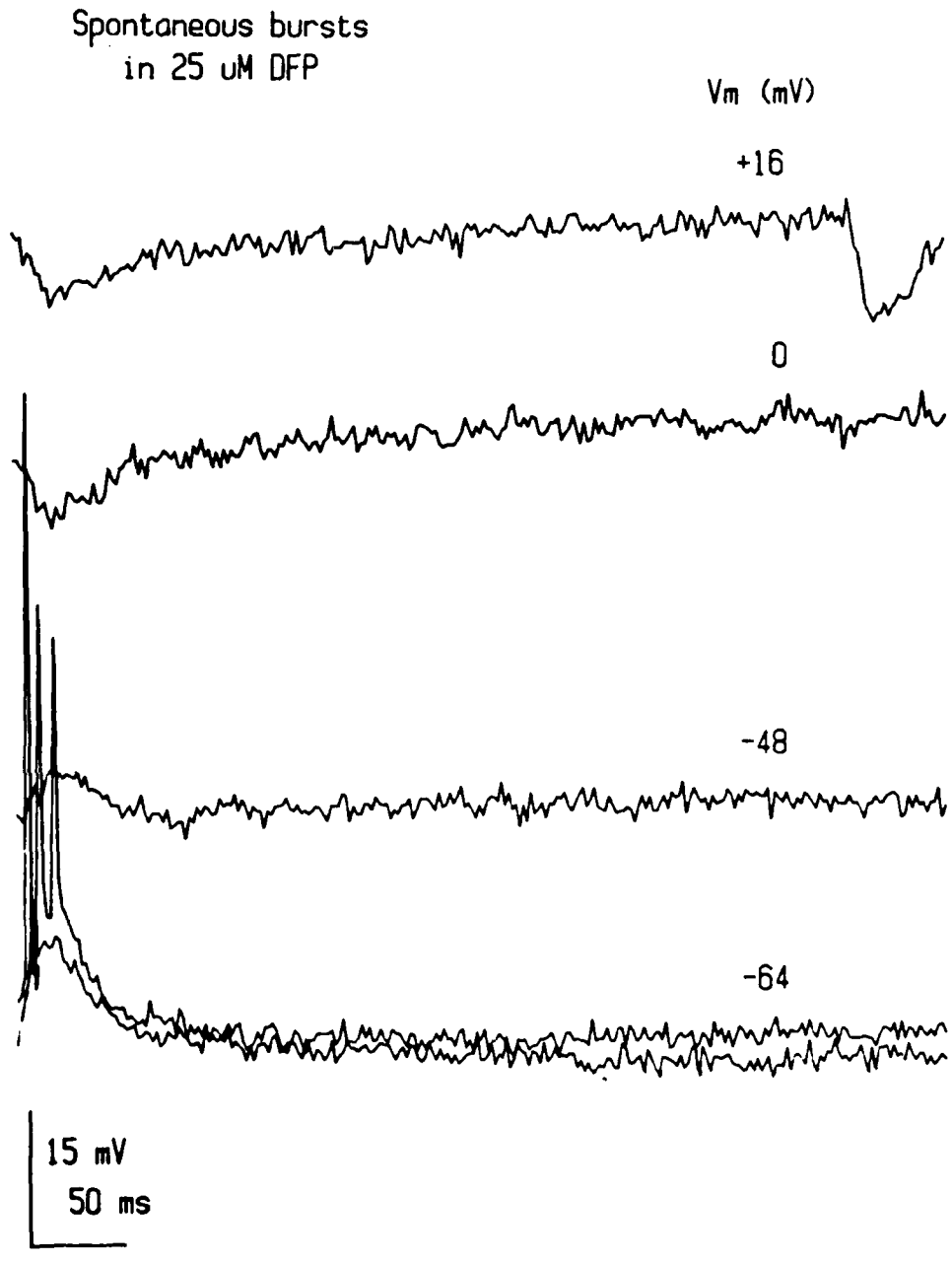


FIGURE 6.
Effects of changing the membrane potential on intracellularly recorded spontaneously occurring DFP-induced PDS's under current-clamp conditions. The early portion of the waveform apparently reversed in polarity near -25 mV (see Figure 7). RMP= 65 mV.

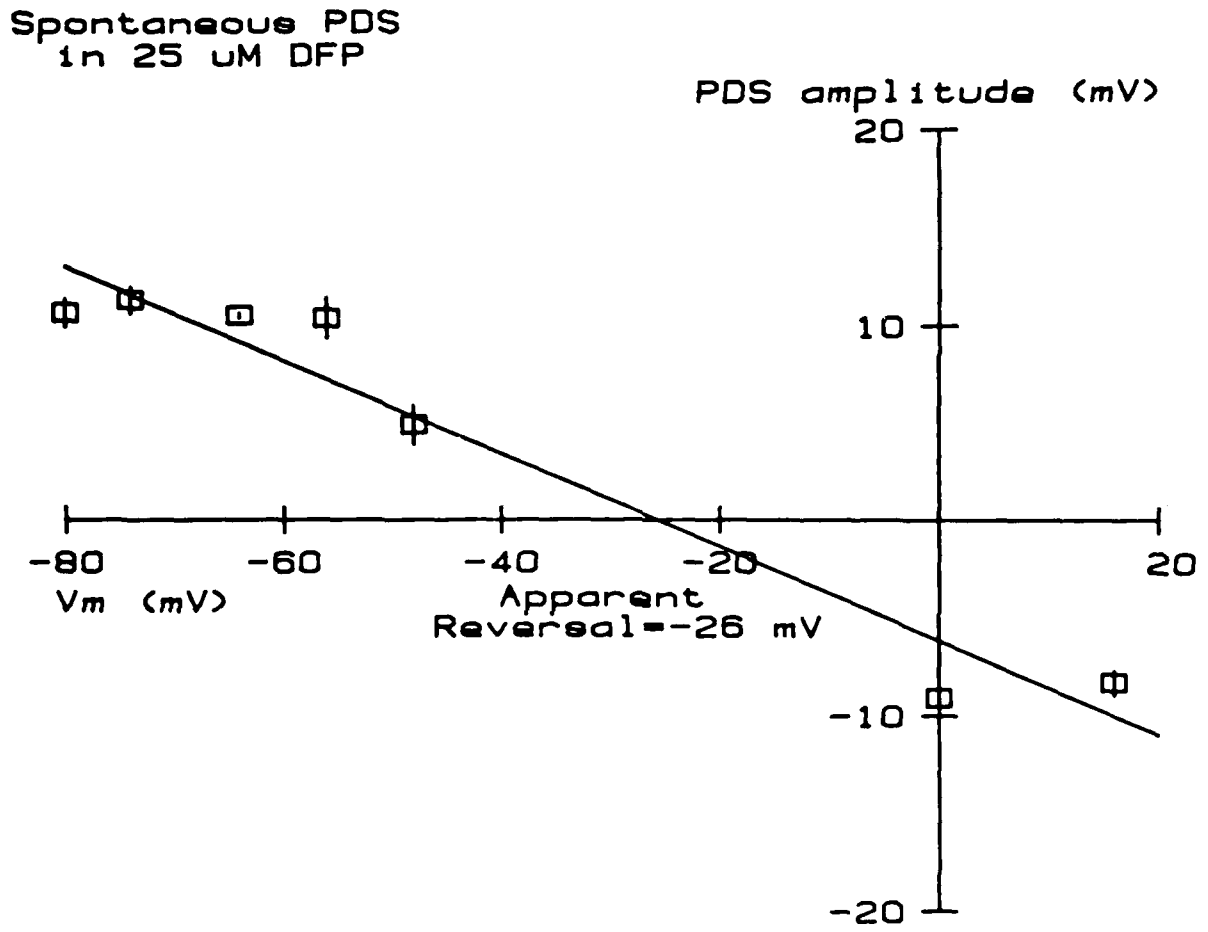


FIGURE 7.

Plot of the initial, peak amplitude of the DFP-induced PDS versus the membrane potential under current clamp. The interpolated apparent reversal potential was calculated to be -26 mV using a least squares fit. The vertical bars shown with the symbols represent the standard error of the mean.

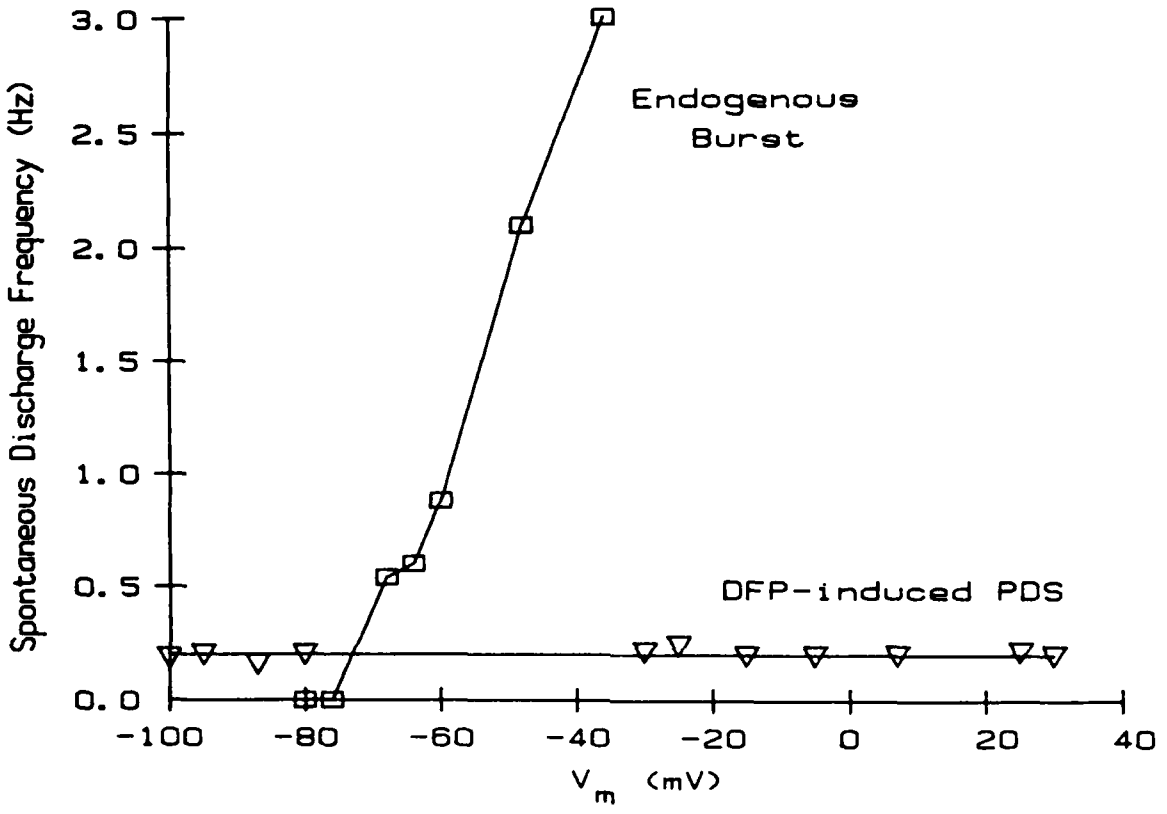


FIGURE 8.

Comparison of the voltage-dependence of the endogenous burst frequency of a CA3 neuron in control saline with the PDS frequency obtained from another neuron in DFP. At membrane potentials more negative than -70 mV and more positive than -30 mV, the endogenous bursts were prevented from being generated; this result indicates a voltage-dependent mechanism. The frequency for the PDS induced by 10 µM DFP was voltage-independent and reflects a synaptically driven process.

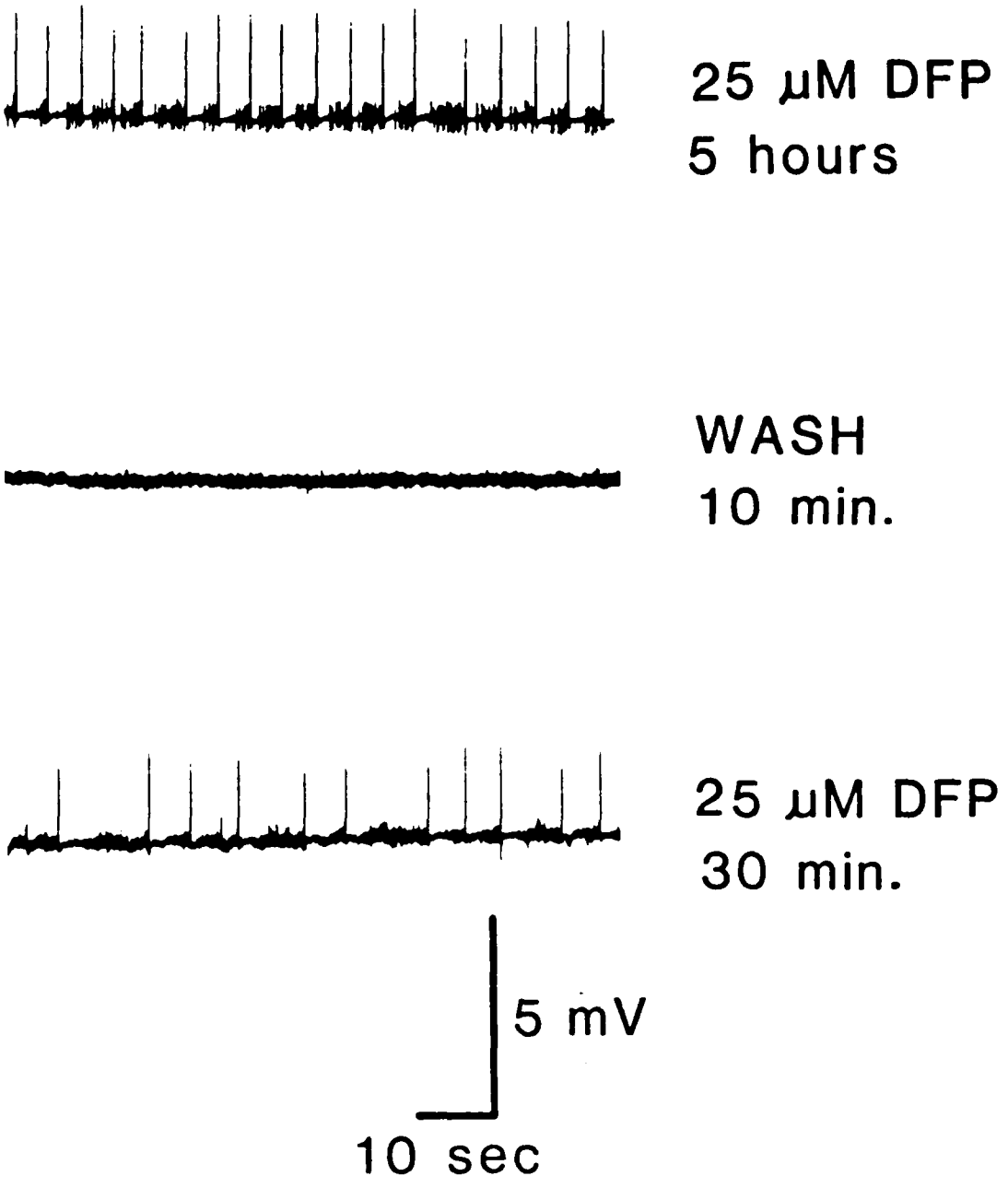


FIGURE 9.

Cessation of DFP-induced periodic discharges upon washout. Top trace shows the spontaneously occurring discharges that were present after a 5 hr exposure to 25 µM DFP. Within 10 min of washout, these discharges were abolished, but resumed within 30 min of the reintroduction of the same DFP-containing saline solution.

TABLE 1

EFFECTS OF VARIOUS PHARMACOLOGICAL AGENTS ON EXTRACELLULARLY RECORDED FIELD ACTIVITY IN THE CA3 REGION OF THE HIPPOCAMPAL SLICE[a]

test agent	conc.(μM)	convulsant[b]	anti-convulsant[c]	depressant[d]
cholinergic agonists:				
acetylcholine	2000	-		-
carbachol	1000	-		-
carbamate anti-ChEs:				
neostigmine	100	-		
	1000	-		
pyridostigmine	100	-		
	200	-		
physostigmine	10	-		
	500	-		
bicyclo-organo-phosphate:				
EBPT	10	+		
ChE reactivator:				
2-PAM	10		-	
	100		-	
anti-muscarinic:				
atropine	1		-	
	10		+	
	1000	-	+	+

[a] In these experiments, 4 to 8 slices were examined over a 30 min period with each bath-applied test agent.

[b] Convulsant activity induced by the test agent was determined by the presence of repetitive field discharges following single orthodromic stimuli delivered to the mossy fiber region at 0.5-1 Hz. Viability of the slices that did not produce convulsant activity in response to the test agent was confirmed by obtaining spontaneous or evoked epileptiform activity with bath-applied 100 μM bicuculline or picrotoxin.

[c] Anticonvulsant activity occurred when spontaneous DFP-induced discharges (25 μM DFP for 30-60 min.) decreased in frequency or were abolished by the test agent.

[d] Depressant activity was determined by observing a reversible diminution of the evoked population response produced by the test agent alone.

Bath-application of the ChE reactivator 2-PAM (10-100 μM) for 30 min had no detectable effect on the epileptiform activity produced by DFP. Similar experimental results were obtained with atropine at 1 μM. However, at high concentrations (10-1000 μM), atropine did abolish these DFP-induced events in a reversible manner. In the absence of a convulsant, this high concentration of atropine also depressed the evoked population response (Hablitz and Lebeda, unpublished observation).

Although not a direct test of the anti-ChE hypothesis, a point should be made regarding convulsant OP agents in general. As listed in Table 1, the bicyclo-OP agent EBPT (10 μM) produced epileptiform activity. This agent thus sets an important precedent in that it represents a class of OP drugs that do not exhibit anti-ChE activity [34] yet do produce abnormal neuronal activity. This observation, in conjunction with the above results, does not support the hypothesis that DFP exerts its epileptiform effects by raising extracellular concentrations of ACh, as a consequence of blocking ChE activity.

D. The Disinhibitory Hypothesis for the DFP-induced PDS

The second hypothesis for the production of epileptiform effects by DFP involves the disruption of inhibitory neurotransmission. For comparative purposes, picrotoxin, the uncompetitive antagonist of GABA [9, 35], was used to evaluate the effectiveness of DFP in abolishing inhibitory synaptic activity. In these experiments, neurons were depolarized to 0 mV in order to observe spontaneously occurring IPSPs. From previous studies [33,36], it is known that the reversal potential of the pure excitatory synaptic event in CA3 neurons is near 0 mV. Since the reversal potential for the inhibitory synaptic event is about -70 mV [37], a physiological separation of the two synaptic process can thus be achieved.

Figure 10 illustrates that spontaneously occurring IPSPs were still present with DFP at 25 μM (n = 3 cells) and some IPSPs may have been larger, in fact, than those occurring in control. This apparent enhancement of IPSP amplitudes will be more rigorously examined in future studies. On the other hand, 10 μM picrotoxin (n = 3 cells) virtually abolished spontaneously occurring IPSPs. Thus, DFP does not induce epileptiform activity by abolishing inhibitory neurotransmission.

The reversal potential of a PDS can also be used as a measure of the relative amount of inhibitory synaptic input that is present with a convulsant. The apparent reversal potential of the DFP-induced PDS was compared to that obtained from the PDS induced by picrotoxin. In Figure 11, 50 μM picrotoxin was bath-applied and the impaled hippocampal neuron was activated by orthodromic stimulation. As with DFP, the picrotoxin-induced PDS was seen to reverse in polarity with depolarization, but the reversal potential for this paroxysmal shift was 25 mV more positive than that for DFP. The corresponding PDS amplitude-membrane potential plot for picrotoxin is shown in Figure 12. A similar value for the reversal potential of the spontaneously occurring picrotoxin-induced PDS was also obtained [38].

As mentioned above, 0 mV is near the reversal potential for excitatory

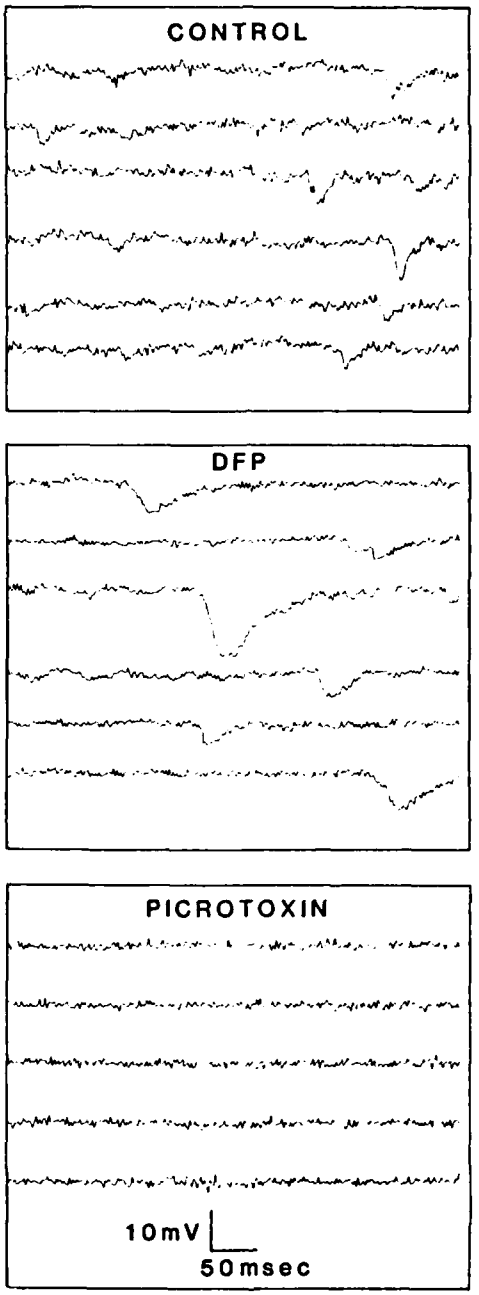


FIGURE 10.

Spontaneously occurring IPSPs during exposure to picrotoxin or DFP. These events were recorded at 0 mV, the reversal potential of the EPSP in these neurons. Records from three different CA3 neurons. In control saline, the amplitudes of these spontaneously occurring IPSPs were about 5 mV; input resistance (RN) = 10 Mohm. 25 μM DFP did not abolish these IPSPs and may have augmented their amplitudes; RN = 15 Mohm. 10 μM picrotoxin virtually abolished these spontaneously occurring events; RN = 10 Mohm.

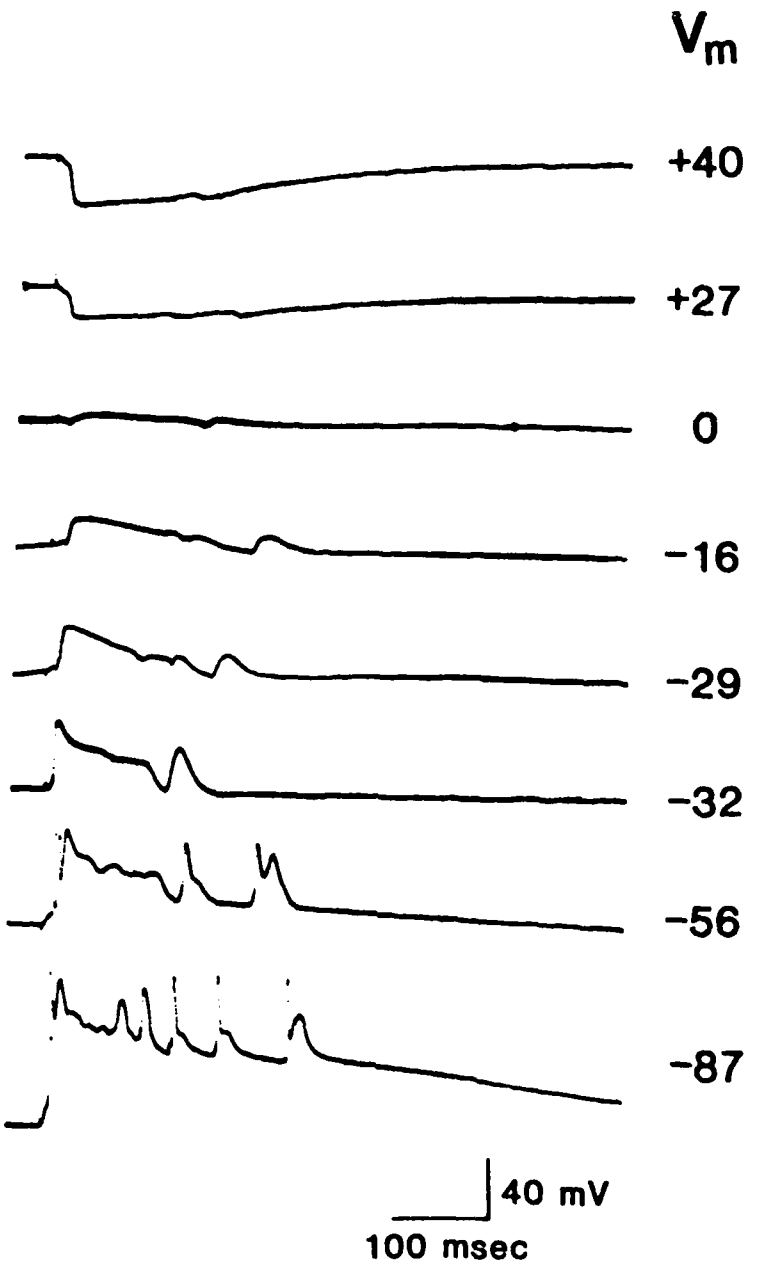


FIGURE 11.

Effects of changing the membrane potential on the picrotoxin-induced PDS. These orthodromically evoked discharges were recorded 30 min after bath-applying 50 μM picrotoxin. The reversal potential for these PDSs, calculated from a least squares fit, was about 0 mV.

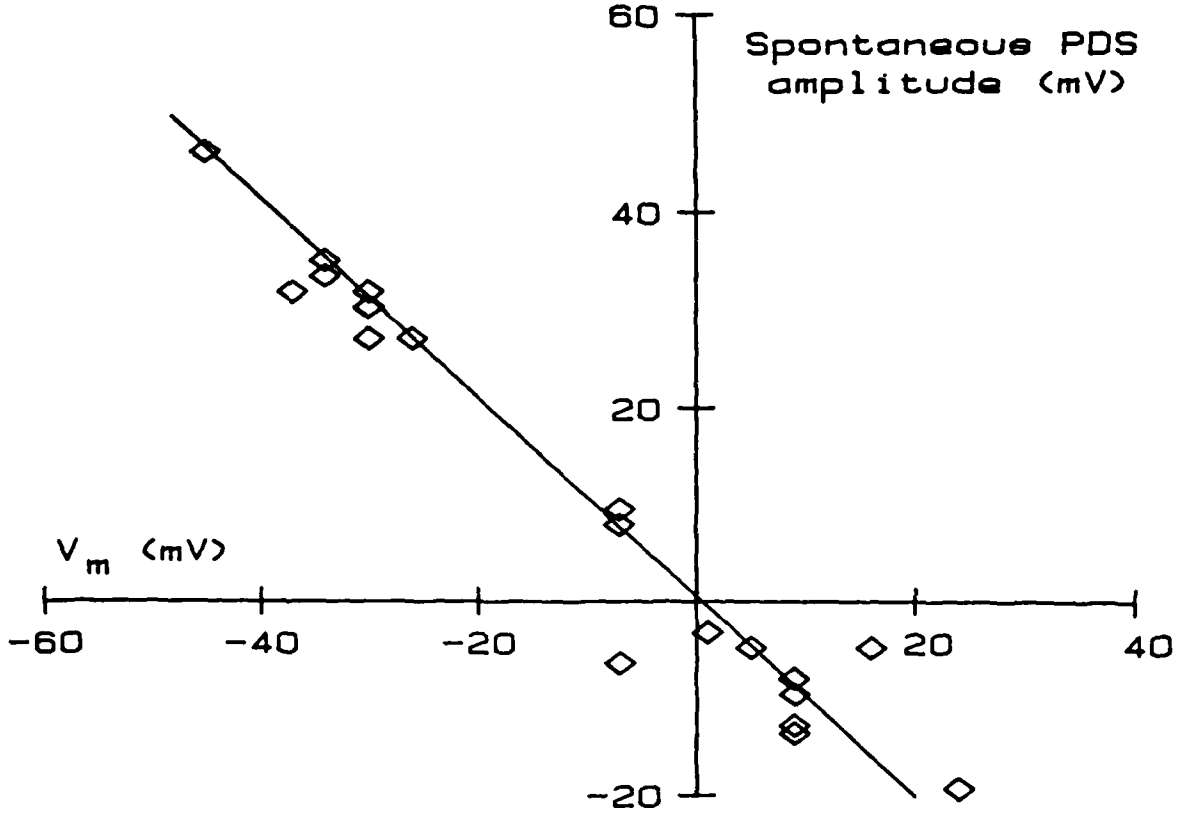


FIGURE 12.

Plot of picrotoxin-induced PDS amplitudes versus membrane potential under current clamp. The interpolated reversal potential was near 0, which is about 25 mV more positive than the value for the reversal potential obtained with the DFP-induced PDS (Figure 7).

events in CA3 neurons, and PDSs induced by convulsants that block GABA-mediated inhibition have approximately the same reversal potential. In keeping with these observations, it is suggested that the PDS induced by DFP has an apparent negative reversal potential, in contrast to the PDS induced by other convulsants, because an inhibitory synaptic component is still present. Although it is possible that the spontaneously occurring IPSPs are affected by DFP, the results so far are not in accordance with the hypothesis that DFP is producing epileptiform activity by a mechanism that abolishes inhibitory neurotransmission.

E. Epileptiform Effects of TEA, 4AP and High Potassium

Concomitant with these DFP studies, other experiments were performed to examine the convulsant effects of two potassium channel blockers, tetraethylammonium (TEA; n = 9) and 4-aminopyridine (4AP; n = 12). As illustrated in Figure 3, 4AP produced the same type of spontaneous, periodic field events as DFP. Similar results occurred with TEA (not illustrated). Further, both of these agents at higher concentrations (5-25 μM 4AP, n = 4; 5 mM TEA, n = 4) produced abnormal waveforms similar to those induced by DFP, including the DC shifts (not illustrated). TEA, however, was at least 100 times less potent than either DFP or 4AP in producing these events.

Since it has been reported that in vivo, extracellular levels of potassium can reach 30-80 mM during these DC shifts [39], studies on the effects of high potassium in the hippocampal slice were initiated. Experiments with saline solutions containing moderately raised levels of potassium (from 6.5 to 10 mM) revealed that this treatment also induced the same spectrum of epileptiform behavior as that produced by DFP and the potassium channel blockers. Raising the potassium concentration to 11-12 mM produced a depression of the evoked synaptic response. Although it has long been known that elevated potassium concentrations can induce abnormal electrical activity, the degree of similarity in the resultant effects with DFP, TEA and 4AP is nevertheless remarkable.

F. Network Modeling

In order to place some of the observed convulsant effects into a more theoretical framework, preliminary modeling of simple network systems was begun. The primary goal was to determine whether synchronous, periodic activity could be generated by a population of non-bursting neurons, i.e., a network of interconnected cells that was not driven by an intrinsic or extrinsic source. Although the latter characteristic has been used in other modeling studies [40], it was deemed important to know whether or not a self-organizing process [41] could emerge from a system of normally quiescent cells.

The network parameters chosen are summarized in Table 2. The only ongoing neuronal activity in the system of 100 randomly connected cells was the presence of spontaneously occurring EPSPs. The EPSP amplitudes were obtained from a Gaussian distribution, while the frequency of their occurrence was generated from a Poisson distribution. The network consisted of simple threshold detectors and had a design similar to the models described

TABLE 2

PARAMETERS FOR THE NEURONAL NETWORK MODEL[a]

parameter	value	dimensions
number of cells	100	-
resting membrane potential	-70	mV
average number of excitatory synapses (Gaussian)	5	-
maximum number of inputs or outputs/cell	10	-
average time between EPSP arrivals (Poisson)	200	msec
average EPSP amplitude (Gaussian)	5-20	mV
standard deviation of EPSP amplitude	2	mV
EPSP decay time constant	10	msec
average EPSP decay time constant (Gaussian)	5	msec
prespike threshold	-50	mV
postspike threshold	-40	mV
action potential duration	1	msec
absolute refractory period	1	msec
decay time constant of threshold after action potential	10	msec

[a] The FORTRAN program NET1 was developed for this simulation. The compiled program was linked to a modified FORTRAN library of arithmetic subroutines that used the floating point hardware.

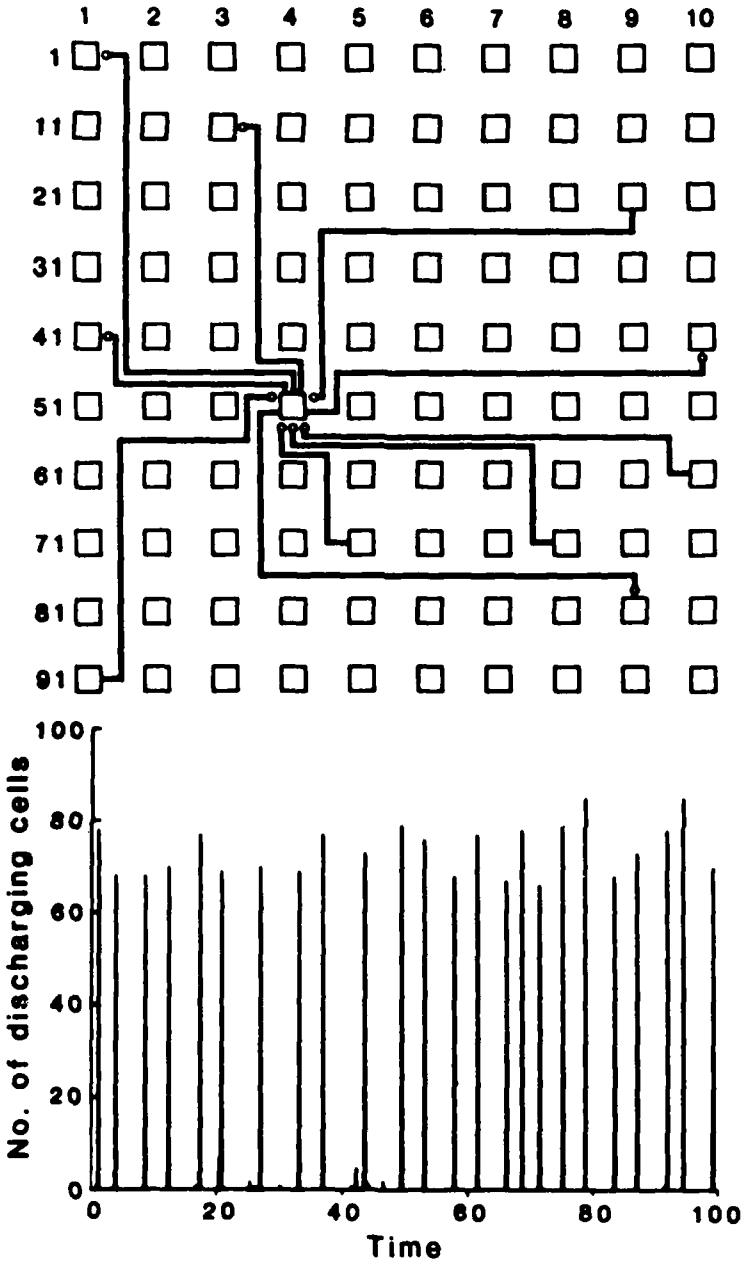


FIGURE 13.

Diagram of model neuronal network and periodic output. The top panel shows an array of model neurons with the synaptic inputs and outputs associated with a typical cell. None of the cells in this model network had pacemaker activity, but acted as simple threshold detectors. Initially, only spontaneously occurring EPSPs were present in the simulation. Summation of these events past a predetermined threshold triggered an action potential. Table 2 contains a list of the parameters used in this model. The bottom panel shows a histogram containing the number of discharging cells as a function of time (in seconds) with an average EPSP amplitude of 5.1 mV.

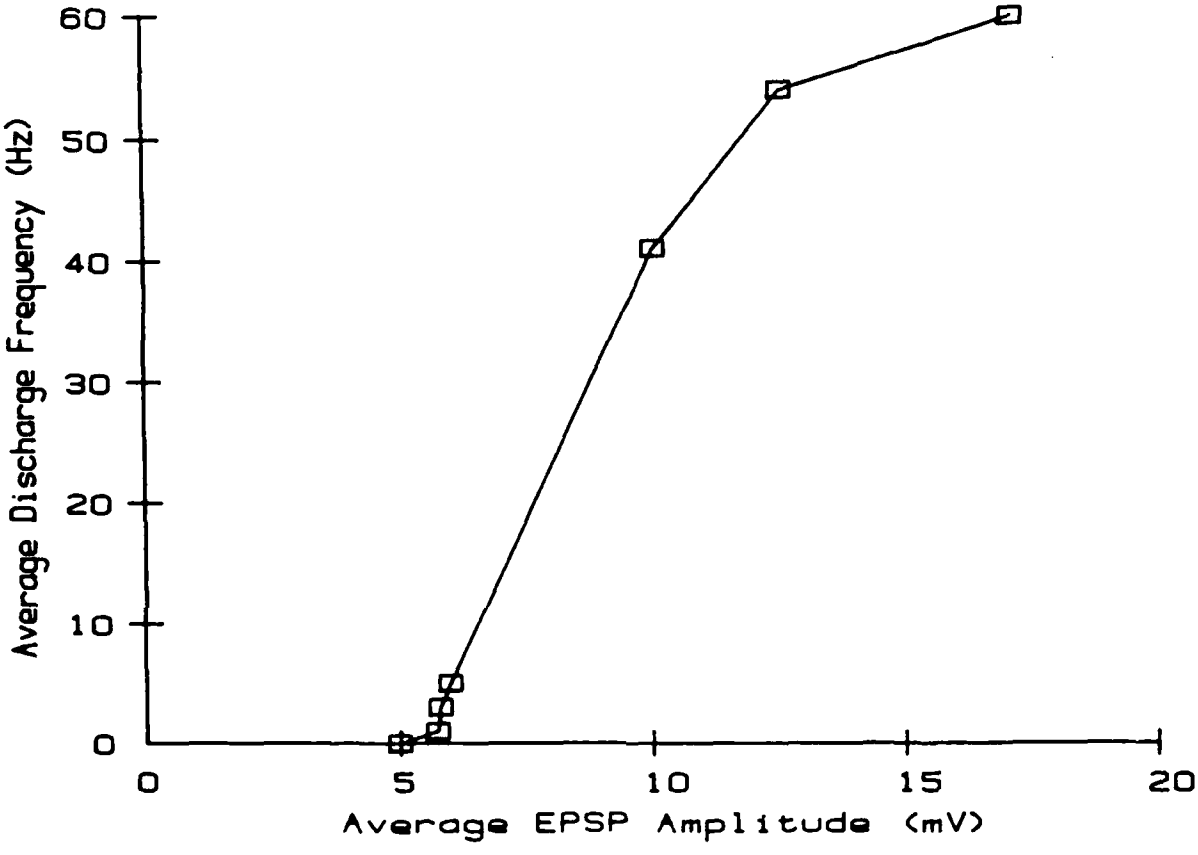


FIGURE 14.
Plot of the theoretical discharge frequency versus the preset average EPSP amplitude. Below an EPSP amplitude of 5 mV, no spontaneous activity was detected. Above 17 mV, the system no longer displayed periodicity, but rather produced a nonsynchronous firing of a majority of cells in the network.

by Dichter and Spencer [42] and Andersen et al. [43]. Linear addition of EPSP amplitudes was used as a crude, first approximation of cellular integration. Once threshold was achieved, an action potential was mimicked by setting the membrane potential to 0 mV for 1 msec. The next msec represented an absolute refractory period in which another action potential could not be triggered. In this randomly connected network (Figure 13), an EPSP was elicited in all the cells postsynaptic to the discharging presynaptic element.

The resultant histogram shown in the lower part of Figure 13 illustrates the total network response using the parameters listed in Table 2 (average EPSP amplitude in Figure 13 = 5.1 mV). Sixty-five to 85% of the cells in the network fired synchronously in a relatively periodic manner. A wide range of output frequencies could be obtained with only small changes in the EPSP amplitude (Figure 14). EPSP amplitudes below 5 mV did not cause the system to oscillate, while amplitudes greater than 17 mV caused most of the cells in the system to become active in a nonperiodic manner. Further studies will be made to determine what connectivity patterns (e.g., number of closed synaptic circuits) are most important for maintaining this PDS-like behavior and for the development of seizure-like activity. It is only through theoretical studies like these that alterations in network activity can be quantitatively monitored as a result of changing subtle neuronal properties such as potassium currents and EPSP amplitudes.

IV. DISCUSSION

The immediate goal of the present study was to characterize the electrophysiological alterations produced by DFP in the CNS. The changes elicited by equipotent concentrations of DFP, TEA and 4AP were remarkably similar, and we intend to pursue the possibility that these agents share a common mechanism of action. The prolonged discharges and DC shifts produced distinguish these agents from those drugs that typically produce only PDS-like activity. Furthermore, the observation that spontaneously occurring IPSPs were not abolished provides further evidence that DFP and the other, similarly acting drugs represent a new class of convulsants. This finding is important, since it has been suggested by several researchers in the field of epilepsy that disinhibition is a prerequisite for seizure activity [44].

A. Characteristics of the DFP-induced PDS

As shown by extracellular recordings, the spontaneous, periodic (0.1-0.3 Hz) discharges induced by low concentrations of DFP (10-25 μM) were superficially similar to those produced by other convulsants (e.g., bicuculline and picrotoxin), which are known to disrupt inhibitory activity mediated by GABA. These types of drug-induced, periodic discharges have been proposed to be the correlates of the interictal spike phenomena seen by the clinical electroencephalographer in epileptic patients [31,32]. Although this DFP-induced effect was fully reversible, DFP differed from the other agents in that it did not abolish spontaneous inhibitory synaptic activity, and may have augmented it (Figure 10). As described below, the apparent negative reversal potential for the DFP-induced PDS provides

additional supportive evidence that an inhibitory synaptic component was still present.

In the recent literature, claims have been made [44,45,46,47,48] that such convulsant-induced discharges are the result of processes that are intrinsic to the pyramidal neurons themselves. This reasoning is based on the fact that both CA1 [49,50] and CA3 [51,52] cells are capable of undergoing a similar form of burst activity under control conditions, because of the existence of voltage-dependent calcium and potassium currents [25,53]. This bursting behavior, however, is not a synchronous phenomenon and is therefore not reflected as a prominent event in the field recordings. In the presence of a convulsant that reduces inhibitory synaptic transmission, it has been argued that EPSPs with normal amplitudes trigger these endogenous currents synchronously in a group of cells [54]. Modeling studies based on this idea, however, have resulted in spontaneous discharge frequencies that are faster than those usually induced with convulsants [48]. The latter point is significant, since a mechanism not requiring endogenously bursting cells can yield repetitive activity over a wide range of discharge frequencies (Figure 14).

As pointed out in previous work [18], a number of predictions can be used to test this endogenous burst hypothesis: 1) If a drug-induced PDS is endogenous in nature, the underlying voltage-dependent event, such as a calcium current, will become smaller in a nonlinear manner either when a voltage-dependent inactivation occurs or as the membrane is depolarized toward the Nernst potential for the particular cation in question; 2) the amplitudes of drug-induced events will be reduced to zero, but never reverse in polarity.

On the other hand, a number of investigators have considered drug-induced PDSs to be synaptic in origin [9,18,32,42,55,56]. Based on this hypothesis, a distinct set of predictions can be made: 1) The frequency of spontaneous discharge will be independent of the membrane potential (Figure 8); 2) the waveform amplitude will be a monotonic function of membrane potential (Figure 7); and 3) the waveform will reverse in polarity as the membrane potential goes beyond the reversal potential of the associated synaptic process (Figures 6 and 7). It is quite evident from the present results with DFP that a synaptic event underlies the PDS.

The reversal potential of PDSs obtained with a variety of convulsants that interfere with inhibitory synaptic transmission (e.g., bicuculline, picrotoxin) is near 0 mV and represents a pure excitatory synaptic process. In control saline, orthodromic stimulation typically results in the appearance of an early EPSP followed by a longer-lasting IPSP [51], which is presumably due to the recurrent excitation of inhibitory interneurons [57]. A calcium-dependent potassium current also contributes to this hyperpolarizing phase [53]. With this group of convulsants, the inhibitory component is greatly reduced, thus revealing the initial, excitatory synaptic response. The resultant reversal potential of this drug-induced PDS is near 0 mV [9,18,38]. Occasionally under control conditions, cells having only an excitatory evoked synaptic response have been observed. The reversal potential for these normal events as well as that for miniature EPSPs [36] has also been close to 0 mV, a result which indicates that the PDS initiated by the blockers of inhibitory transmission is an excitatory

synaptic phenomenon.

In contrast, the apparent reversal potentials for the DFP-, TEA- and 4AP-induced PDSs were about 20 to 30 mV more negative under current-clamp conditions than the reversal potentials obtained with the convulsants that disrupt inhibitory neurotransmission. This range of apparent values for the PDS reversal potential is dependent on the manner in which the measurements are performed. In the present study, the initial peak amplitude of the waveform was chosen for the measurement, but other methods, such as measuring at a fixed time after the onset of the PDS, may yield quantitatively different results. Notwithstanding this problem, the apparent negative reversal potentials for the PDSs induced by DFP and the other convulsants may be the result of a residual, inhibitory component contaminating the measurement of the reversal potential of the early, excitatory synaptic response; i.e., the time course of the excitatory process is abbreviated and its true amplitude obscured by the subsequent inhibitory phase. It is anticipated that in future voltage-clamp studies, the time course of these two synaptic events will be temporally resolved and that the correct reversal potential for the PDS induced by DFP and the other drugs also will be near 0 mV. Nevertheless, the apparent negative reversal potential obtained with conventional techniques was one of the first indications that this group of convulsants acted differently from those agents studied previously.

B. Prolonged DFP-induced Discharge

At higher concentrations, DFP initiated a secondary discharge pattern superimposed on the previously seen periodic events. This new waveform lasted 2-5 times longer than the PDS and may represent a transitional phase that will lead to an ictal-like phenomena. Similar discharges were also seen with higher concentrations of TEA (5 mM) and with 4AP (5-25 μM) in the present study, and with 4AP in olfactory cortex slices by other investigators [58]. The variability of occurrence in the hippocampus of these longer, less frequent events, however, precludes a detailed study of the cellular basis of this epileptiform discharge at this time. It should be emphasized that the previously examined convulsants, which disrupt inhibitory synaptic activity, rarely produced this type of abnormal discharge in vitro.

C. DFP-induced DC Shift

At the highest concentrations of DFP examined, the repetitive field discharges were replaced by relatively infrequent, large (10-20 mV) DC shifts in the extracellularly recorded potential. Similar long-lasting events (1-2 min) have been reported to occur with penicillin in vivo [59] and may be related to the chemically induced spreading depression of Leao [39,60], since evoked responses could not be elicited during these shifts. High levels of potassium also have been observed extracellularly (up to 30-80 mM; [39]), and may serve, along with other processes such as the influx of calcium ions, to initiate spreading depression.

Nonsynaptic mechanisms for these DC shifts also have received attention. Dudek and his collaborators [61,62] have demonstrated that in the hippocampal slice, excessive stimulation can trigger these events at a time

when synaptic activity is reduced. It has been reported that solutions low in calcium, which tend to reduce synaptic transmission, are themselves sufficient to produce spontaneously occurring DC shifts [63]. An additional observation in support of this conclusion comes from preliminary work with neocortical slices (Hablitz and Weiss, unpublished observations). In this preparation, both penicillin and picrotoxin were able to generate only sporadic PDS-like discharges but were powerful inducers of DC shifts. By using two separate extracellular recording electrodes, it was found that, although repetitive field discharges were delayed by only tens of milliseconds, the DC shifts were more than 5 sec apart, a result which is consistent with the idea that one or more substances (e.g., potassium or the excitatory amino acid glutamate) are diffusing across the preparation to cause the large, long-lasting responses [64]. It is not clear at present why the neocortical preparation is more likely than the hippocampus to produce these abnormal responses. It is possible that the network connectivity pattern is the critical, distinguishing feature of these two areas of the brain. Future network modeling studies will investigate this possibility.

D. Mechanisms for the DFP-induced PDS

As stated in the Introduction, two hypotheses were originally considered to account for this DFP-induced epileptiform event: the anti-ChE mechanism and the disruption of inhibitory neurotransmission. The predictions made from these two hypotheses were tested, and the results were inconsistent with both ideas.

The anti-ChE mechanism of action for the DFP-induced PDS is not supported by the following evidence from the in vitro hippocampal preparation: 1) The DFP action was abolished upon washout (Figure 9); 2) similar effects were not observed with the carbamate anti-ChEs or with cholinergic agonists (Table 1); 3) the DFP-induced PDS was not antagonized by the ChE reactivator 2-PAM or by the lowest concentration (1 μM) of atropine (Table 1). The blockade of the DFP-induced spontaneous discharges with 10-1000 μM atropine was not considered to be due to its anti-muscarinic effect but, rather, may reflect a nonspecific depressant activity. Since only a limited number of experiments were performed with this assortment of agents, additional trials will be conducted to confirm these initial results. Furthermore, other muscarinic blockers and ChE reactivators will be evaluated in future studies.

Additional results also suggest that a disruption of inhibitory synaptic transmission is not the primary effect of DFP. Inhibitory synaptic activity was not abolished, since spontaneously occurring IPSPs were still observed in the presence of this agent (Figure 10). This hypothesis was further questioned when higher concentrations of DFP produced additional types of abnormal waveforms that have not been seen previously with GABA antagonists. Again, additional study is required to completely eliminate this mechanism from consideration. For example, the frequency of spontaneously occurring IPSPs may be reduced with DFP. Furthermore, the apparent increase in IPSP amplitude may reflect a partially developed compensatory mechanism to counteract some as yet unidentified drug-induced alteration in inhibitory synaptic function.

The present results, however, are complemented by preliminary findings obtained with cultured chick cerebral neurons [65,66,67]. In this preparation, DFP did not affect labeled-GABA binding or GABA-activated chloride flux (Barnes and Thampy, unpublished observations). In contrast, the bicyclo-OP convulsant EBPT (Table 1; [68]), which does not possess anti-ChE activity [34], significantly reduced the GABA-activated chloride flux in this preparation without affecting GABA binding [67]. Indeed, this agent is at least 10 times more potent than picrotoxinin [69] in blocking GABA-activated chloride channels. It remains to be determined whether convulsant OP anti-ChEs other than DFP display this type of channel blocking activity.

A third hypothesis was developed after experiments showed that two potassium channel blocking agents, TEA [19,20] and 4AP [21], produced a DFP-like spectrum of epileptogenic responses. Although it has been known for some time that 4AP is capable of inducing seizure activity [70], only recently published studies show results comparable to those events initiated by DFP [58]. It has also been suggested that sarin may alter ionic conductances (perhaps involving potassium), thereby producing the observed desynchronization of respiratory center unit discharges before the reduction of phrenic nerve-diaphragm activity [71,72].

Several outward currents (some of which may be associated with other ions besides potassium) have been identified in hippocampal pyramidal cells, e.g., a delayed rectifier (D. Johnston, unpublished observations); a calcium-dependent outward current [53]; an early, fast outward current [73]; Q-current [74]; M-current [75]. These currents may be pharmacologically separable and characterized by their voltage-dependent kinetics. For example, by examining tail currents, Brown and Griffith [53] demonstrated that TEA substantially reduces a calcium-dependent potassium current. Evidence from other studies [73] indicates that 4AP causes a reduction of an early, fast outward current. The detailed mechanisms responsible for the high potassium-induced effects may also include a decrease in the electrochemical driving force and, hence, a reduction of the total outward current. The effects produced by DFP on these currents will be examined in detail in future experiments (see Recommendations for Future Research [Section V.], below).

If a blockade of one or more potassium channels is important in the convulsant action of DFP, the expected physiological consequences may involve alterations in synaptic transmission. It is possible that the reduction of one of these potassium currents could prolong the presynaptic action potential waveform and thus enhance the postsynaptic response. Evidence supportive of this action has been obtained, using 10 μM 4AP in the olfactory cortex slice [58], in which a 3-fold change in the duration of the presynaptic population spike occurred along with augmented postsynaptic potentials. It is presently not known whether a reduction of only one of the several outward currents listed above could prolong action potentials at presynaptic nerve terminals. If a presynaptic action for DFP or the similarly acting convulsants were to occur, such a nonspecific effect would also be expected to lead to an augmentation of the IPSP waveform, a result that has been observed in the hippocampal slice [76; see also Figure 10].

Observing a generalized increase in synaptic transmission would be in keeping with the idea that epileptiform activity reflects a system in disequilibrium [77]. In the hippocampus, disruption of a delicate balance between excitatory and inhibitory synaptic function would result if inhibition were abolished. Similarly, it can be envisioned that seizures would become manifest if there were a net increase in excitatory synaptic activity. This possibility is presently being considered, and a detailed analysis of excitatory and inhibitory events will be conducted in future experiments.

E. Effects of ACh and Anti-ChEs In Vivo

These postulated changes in outward, potassium-mediated currents caused by DFP and several other convulsants may indeed be an important mechanism of epileptiform action. However, from the wealth of evidence outlined below regarding the convulsant effects induced by anti-ChEs and by drugs acting on cholinergic transmission, it is conceivable that DFP may exert additional, related effects by a cholinergic mechanism. Investigations concerned with the changes produced by anti-ChE in the CNS have been conducted since the 1860s and have generated a plethora of pharmacological data. Surveys of the older literature have been compiled by Machne and Unna [78] and Karczmar [79], while a detailed overview of the more recent investigations with OP anti-ChEs has been gathered by Ellin [80].

It is well known that, depending on the experimental conditions, acetylcholine can, in a dose-dependent manner, either produce seizures or cause depression in intact animals. The pioneering work of Moruzzi [81] demonstrated that intra-arterial or topical administration of low doses of ACh caused excitatory responses, whereas higher doses or ChE pretreatment caused a marked inhibition of cortical activity that was sufficient to abolish drug-induced seizures.

Disparate results, however, have been reported concerning the changes in electrical activity induced by a variety of anti-ChEs. For example, intravenously applied eserine prevented curare- or penicillin-induced seizure activity, whereas neostigmine had no effect [82]; this result, however, may be due to differences in penetrating the blood-brain barrier with these agents. Based upon this and other examples of contradictory data, it has been suggested that the convulsant mechanisms involved with anti-ChEs may not be implicated directly with cholinergic transmission [78].

More recent studies support this assertion. The protective effects of the ChE reactivator HI-6 against twice the LD50 of soman occur at a time when both blood and brain ChE are inhibited [83]. It was concluded by the authors that the oxime protects against the lethal effects of soman at sites associated with central cholinergic synapses, besides on the ChE molecule, or possibly at noncholinergic sites.

Drugs affecting a multitude of areas within the CNS may cause the development of seizure activity through various interactions, whereas these drugs may have only subconvulsant effects upon an isolated region, such as the hippocampus. It is therefore reasonable to focus attention upon specific areas within the CNS to help clarify this assortment of results.

The role of ACh within the hippocampus has recently become the subject of intense investigation. A major portion of the cholinergic input to the hippocampus comes from the septal nuclei. This conclusion is based on the findings that lesions produced in the septal area cause an almost complete loss of ChE and cholinacetyltransferase activity [84,85]. This loss of activity is in accordance with the decrease in the theta rhythm following septal lesions [79]. Stimulation of the intact septum has been associated with ACh release [86,87,88], and it has been demonstrated that the enhancement of theta activity by physostigmine [89] may be due to a mixed nicotinic and muscarinic action [90]. Furthermore, septal projections have been traced to the stratum oriens in the hippocampus [84].

Several lines of evidence indicate an excitatory function for ACh. Stimulation of the septal region in vivo, however, does not cause a significant change in the field response in either CA1 or CA3 subregions [91]. The small but detectable changes induced in vitro in CA1 cells with repetitive stimulation of the stratum oriens in the CA3 area can be mimicked with bath-applied ACh, facilitated with eserine and blocked with atropine [22]. The hippocampal response to septal stimulation in vivo, in contrast, has a facilitatory effect on both fimbrial and commissural-induced events in the CA1 field [91]. Modulatory effects of ACh have also been suggested to account for both increases and decreases observed in EPSP amplitudes [7]. In addition, inhibitory events are reduced by 10 μM carbachol in the hippocampal slice [92]. From these observations, it is readily perceived that ACh operates by more than a single mechanism in the hippocampus.

The mechanism by which ACh enhances EPSP amplitudes probably occurs by an increase in the postsynaptic membrane input resistance [5,6,8,92] and has been speculated to occur as a result of a decrease in the M-current [22,91]. Additionally, the cholinergic input has been suggested to block a calcium-dependent potassium current, since ACh has been demonstrated to reduce the prominent afterhyperpolarization in CA1 cells [22,93]. That an agonist can regulate a voltage-dependent current is not a novel suggestion, since it was first demonstrated in molluscan neurons that serotonin can enhance a calcium-mediated current [94].

Although in the present study a reduction in the total outward current is postulated to be involved in the convulsant mechanism of action of DFP, 4AP and TEA, it is not clear whether the blockade of only a single species of potassium currents is sufficient to have generated all the types of epileptiform discharges that were observed. Furthermore, in the development and testing of potential antidotal agents, it will be vital to know whether the same set of channels are being affected by this new class of convulsants.

An important discrepancy remains to be addressed. The present in vitro study demonstrated that, in contrast to DFP, the carbamate anti-ChEs and the two cholinergic agonists tested were not effective in producing epileptiform discharges, while the literature is replete with studies which show that the latter agents can produce seizure activity. Recent investigations in this laboratory, using the hippocampal slice, however, may shed light on this problem.

These studies indicate that pronounced activity-dependent effects can

be induced by a muscarinic agonist, bethanechol, and a muscarinic antagonist, atropine (Hopkins and Johnston, unpublished observations). Although atropine was shown in the present study to exert effects only at high (Table 1), this drug, at 1-10 μM, was observed to enhance long-term potentiation (LTP) of the field response after repetitive, orthodromic stimulation. Moreover, similar concentrations of bethanechol completely blocked the development of LTP. After repetitive stimulation, however, bethanechol alone produced repetitive discharges in response to single, orthodromic stimuli. Additional evidence for an activity-dependent cholinergic mechanism comes from recent work in which focal application of ACh (20 mM) in the hippocampal slice prolonged penicillin-induced PDSs [93]. Further examination of these activity-dependent effects with the other cholinergic agonists and anti-ChEs may help to reconcile the data from in vitro and from in vivo studies in which the relative levels of spontaneous neuronal activity in different supraspinal areas could modulate a drug's mechanism of action.

Our present working hypothesis is that DFP induces the PDS by a novel mechanism, distinct from its well-known anti-ChE activity, possibly by blocking one of the potassium channels described above. In a modified scheme, DFP may, in addition, be acting directly at a cholinergic synapse by blocking an associated ACh-gated ionic channel. An analogous system is the neuromuscular junction where DFP [95] blocks ACh-activated channels in a manner that is kinetically related to the belladonna alkaloids and TEA [96,97]. At present, a DFP-induced alteration in inhibitory synaptic function is not supported by the available evidence, but more detailed, quantitative studies are required to thoroughly evaluate this hypothesis.

None of these postulated mechanisms can be ruled out in explaining the DFP-induced prolonged discharges or the DC shifts. It is possible that the level of neuronal activity within a network can play a pivotal role in determining the net effect of a particular drug. The spectrum of epileptogenic effects caused by DFP may indeed be the result of an intricate relationship among the several mechanisms discussed and some type of use-dependent process involving cholinergic neurotransmission in the CNS.

V. RECOMMENDATIONS FOR FUTURE RESEARCH

The findings obtained with DFP provide a foundation for future studies with other OP anti-ChE agents. While conducting the present set of experiments, a new hypothesis was formulated regarding the convulsant mechanism of DFP. It is our intention to test this idea thoroughly as well as to perform more extensive tests of the originally considered hypotheses to determine whether more than one mechanism is involved in the generation of the epileptiform discharges by DFP and the other, similarly acting convulsants.

1. In order to evaluate in more detail the effects on synaptic transmission of DFP and related convulsants, a quantitative examination of the inhibitory and excitatory synaptic events (i.e., frequency, amplitude and time course) will be conducted.

2. Further trials will be conducted with the previously used, as well as other, untested, cholinergic agonists, antagonists, ChE inhibitors and reactivators to explore the possible cholinergic involvement in OP anti-ChE-induced epileptiform activity.

3. Voltage-clamp experiments will be initiated to examine the effects produced by these agents on outward currents measured from neurons in hippocampal slice preparations.

4. To examine these outward currents in finer detail, the isolated hippocampal neuron preparation will continue to be developed for future experiments, using the patch-clamp technique.

5. The network modeling studies will be continued and updated to incorporate those physiological parameters that are involved in the production of epileptiform activity.

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