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ELECTROPHYSIOLOGICAL EFFECTS OF DRUGS KNOWN TO AFFECT ACETYLCHOLINESTERASE AND ITS INHIBITION ON NEURAL MECHANISMS OF RAT SEPTAL NUCLEI, IN VITRO

Annual Report

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The purpose of our work is to explore the possibility that anticholinesterase drugs and acetylcholinesterase may produce effects on cholinergic and non-cholinergic systems by mechanisms which may or may not involve inhibition of cholinesterase. This project has utilized an in vitro brain slice preparation from rats. This slice contains the septal nuclei, which contain both cholinergic and noncholinergic neuronal networks. We employ intracellular electrophysiological techniques to analyze the action of endogenous transmitters and of drugs applied exogenously. Our level of analysis is at the individual neuron's cell membrane.

Our results demonstrate that pyridostigmine does have direct actions that can not be interpreted as being solely due to inhibition of cholinesterase.. [illegible] neurobiology; cholinergic agents; pharmacokinetics; (KT)

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SUMMARY

The purpose of our work is to explore the possibility that anticholinesterase drugs and the enzyme, acetylcholinerase itself, may produce effects on cholinergic and non-cholinergic systems by mechanisms which may or may not involve inhibition of acetylcholinesterase. This project has utilized an *in vitro* brain slice preparation from male rats. This slice contains the septal nuclei, which contain both cholinergic and non-cholinergic neuronal networks. We employ intracellular electrophysiological techniques to analyze the action of endogenous transmitters and of drugs applied exogenously. Our level of analysis is at the individual neuron's cell membrane.

Our results demonstrate that pyridostigmine does have direct actions that can not be interpreted as being solely due to inhibition of cholinesterase.

FOREWORD

Citations of 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 Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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BACKGROUND

Cellular electrophysiological and pharmacological studies of the limbic system have dealt primarily with the hippocampus. A limbic area that has received far less attention, other than the early *in vivo* reports by DeFrance *et al*. (1,8) and McLennan and Miller (2), is the septal nuclei. Although these early investigations have contributed valuable information about the interactions that occur between the hippocampus and septum (Fig. 1), little is known at the cellular level about the mechanisms that control and/or modulate activity within the septal nuclei or activity between the hippocampus and the septum.

Input from the hippocampus has been described as being due to an excitatory amino acid acting on a kainate/quisqualate-type receptor localized on dorsolateral septal nucleus (DLSN) neurons (3). We have confirmed these results (unpublished observations) and have also described the inhibitory influence of GABA acting on both $GABA_A$ and $GABA_B$ receptors located on the same DLSN neurons (4-6). As a result of these studies, we have established an *in vitro* system in which we can induce a series of three orthodromic responses by applying a focal stimulus to fimbrial afferents coursing from the hippocampus and terminating on dorsolateral septal nucleus neurons (Fig. 2). The orthodromic responses consist of: A) an initial, excitatory-amino-acid-activated, excitatory postsynaptic potential (EPSP); followed by B) a rapid GABA-activated, chloride-mediated, bicuculline/picrotoxin-sensitive, fast inhibitory postsynaptic potential (ipsp); and finally C) a slow, putative GABA-activated, potassium-mediated, bicuculline/picrotoxin-insensitive, late hyperpolarizing synaptic potential (LHSP).

Our working hypothesis is based upon the earlier observations by VanMeter *et al*. (7) that organophosphate and anti-acetylcholinesterase (anti-AChE) agents can induce convulsions by a mechanism different from that involving inhibition of cholinesterases and accumulation of acetylcholine (ACh). We intend to determine if a reversible anti-AChE, pyridostigmine, and/or an irreversible anti-AChE, soman, act directly, i.e., by an action which can not be explained by AChE inhibition, to affect DLSN neuronal spontaneous or synaptic activity.

APPROACH TO THE PROBLEM

Our approach has been to record intracellularly from neurons identified anatomically within specific regions of the septal nuclei.

Rat septal brain slices were prepared as previously described by Stevens et al (6). Male Sprague-Dawley rats weighing between 150-250/g were decapitated and the brain rapidly dissected out and placed in cold Krebs solution of the following composition (in mM): NaCl, 117; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$ 1.2; NaH_2PO_4, 1.2; glucose, 11.5; $NaHCO_3$, 25; and pre-oxygenated with 95% O_2 and 5% CO_2. Transverse blocks of tissue containing the septum were serially cut on a Vibroslice (Campden Instrument) into 500 μm thick sections. A single slice was placed in a recording chamber and superfused with oxygenated Krebs solution warmed to 32 $\pm$ 1°C. Conventional intracellular recording methods were employed using glass micropipettes (75-110 M Ω) filled with 4M potassium acetate. Hyperpolarizing current pulses (0.1-0.15 nA) were injected into the recording pipette via commands generated through an Axoclamp (Axons Instrument) amplifier. Orthodromic stimuli were delivered with square wave pulses (5 to 20 V, 0.15 ms) via concentric bipolar stainless steel electrodes placed focally within the DLSN. Signals were displayed on an oscilloscope and chart recorder, and stored on videotape for offline analysis.

Thus far we have limited our recordings to neurons in the DLSN. We have examined the action of pyridostigmine, 10^{-8} to 10^{-4} M, applied by superfusion, while monitoring three principal parameters:

1) Its effects on active and passive membrane properties of individual neurons
2) Its effects on orthodromic synaptic activity recorded from individual DLSN neurons
3) Its effects on spontaneous activity of individual DLSN neurons

Still to be examined are the effects of pyridostigmine on:

1) Bicuculline-induced epileptiform activity
2) Responses induced by GABA or baclofen applied exogenously.

We have applied pyridostigmine in the presence of atropine and/or mecamylamine to determine the type of cholinergic receptor that may be involved in the action of pyridostigmine.

Although our main approach is electrophysiological, we intend to collaborate with Dr. Gilbert Hillman in our Pharmacology Dept. to examine the neuronatomy of our slices with his staining and image analysis techniques after we have done the electrophysiological experiments.

RESULTS AND DISCUSSION

1) Presence of ACh receptors on DLSN neurons

Based on the earlier studies with atropine by DeFrance et al. (8), a suggestion had been made that cholinergic interneurons might reside within the DLSN. More recent receptor-ligand binding studies by Segal et al. (9) demonstrate that both nicotinic and muscarinic binding can be measured in the DLSN. Although these two pieces of data support each other, there is no histochemical or immunohistochemical evidence using either ChE-staining or choline acetyltransferase (CHAT) immunochemical procedures to support the proposal that ACh or an anti-AChE agent would produce any pharmacological action to induce convulsant-like activity from DLSN neurons.

On the other hand, there is a good deal of anatomical, physiological and pharmacological data describing the well-known cholinergic pathway from the basal forebrain nuclei (n. diagonal-band of Broca and medial septal n.) to the hippocampus (10). We are speculating, that these cholinergic neurons from the basal nuclei send collaterals to the DLSN as their axons pass through to the hippocampus. Such a possibility would explain the two pieces of data we have cited above and our results as cited below.

2) Actions of pyridostigmine and carbachol on DLSN neurons

Our initial studies have been aimed at determining whether pyridostigmine alters spontaneous and/or synaptic activity recorded from DLSN neurons. Figure 3A depicts the results obtained with the lowest concentration of pyridostigmine (2.5×10^{-7} M) that demonstrates a direct inhibitory action on slow spontaneous activity recorded intracellularly from a DLSN neuron. A more dramatic action, but with similar rapid onset and offset, is seen when a higher concentration, 1×10^{-6} M, of pyridostigmine is applied (Fig. 3B). Finally, Fig. 3C depicts the action of carbachol (10^{-6} M), which was chosen for its similarities with respect to degree of hyperpolarization, inhibition, onset and duration to the comparable concentration of pyridostigmine.

Since we were to determine whether pyridostigmine acts by a mechanism that could not be explained solely by inhibition of AChE, we examined its action in the presence of the muscarinic antagonist atropine (10^{-6} M) and the nicotinic antagonist mecamylamine (10^{-5} M). Although such experiments do not rule out the possibility that the action of pyridostigmine may be due to inhibition of AChE, they do discriminate the type of receptor, muscarinic vs. nicotinic, that may be activated if a cholinergic mechanism is involved. Our results (Fig. 4) suggest that pyridostigmine produces a membrane hyperpolarization, which diminishes the effectiveness of synaptic inhibitory responses by

activating a nicotinic -- not muscarinic-postsynaptic cholinergic receptor. We then determined whether this hyperpolarizing action was due to a direct postsynaptic receptor mediated effect (i.e., not due to potentiation of endogenously released ACh by pyridostigmine's anti-AChE effect) by testing pyridostigmine when synaptic transmission was depressed or completely blocked by a superfusion solution containing zero calcium and elevated magnesium (6mM). Under these conditions, evoked synaptic transmission was blocked (Fig. 5); nonetheless, the membrane was hyperpolarized. Even under these conditions, the neuron displayed nonsynaptic rhythmic bursting behavior. It is possible that even though we have eliminated action potential mediated synaptic release with TTX or zero Ca^{++}/high Mg^{++}, spontaneous cholinergic release is still ongoing. This spontaneous cholinergic release may be potentiated by pyridostigmine's anti-AChE action and thereby complicate our interpretation of the results. However, we do not believe this is a major problem, because of the rapid reversibility of the phenomena we observe. If cholinesterase were inhibited significantly, then a much longer recovery time would be expected. Nonetheless, we will keep this possibility in mind when we compare pyridostigmine with soman.

At present, our sample size is still relatively small, N = 25 neurons, each from a different brain slice. The table below summarizes the general membrane potential changes we have observed.

Membrane Effect	Number of DLSN Neurons
Hyperpolarization (2 to 10 mV)	15
No Effect	8
Depolarization (2 to 6 mV)	2

In all 25 instances, during or immediately following application of pyridostigmine over the concentration range of 2×10^{-7} to 1×10^{-4} M, each neuron exhibited an increased excitability as evidenced by induction of busting activity. During this treatment period the fast ipsp and late hyperpolarizing synaptic potential were depressed.

CONCLUSIONS

Our data demonstrate that pyridostigmine produces a direct postsynaptic membrane action on rat DLSN neurons. This action results in membrane hyperpolarization, depolarization or no change in potential, and always produces increased membrane excitability with bursting activity. We believe this action of pyridostigmine is mediated by direct activation of a nicotinic cholinergic receptor, since it can be blocked by mecamylamine but not atropine and is readily and rapidly reversible.

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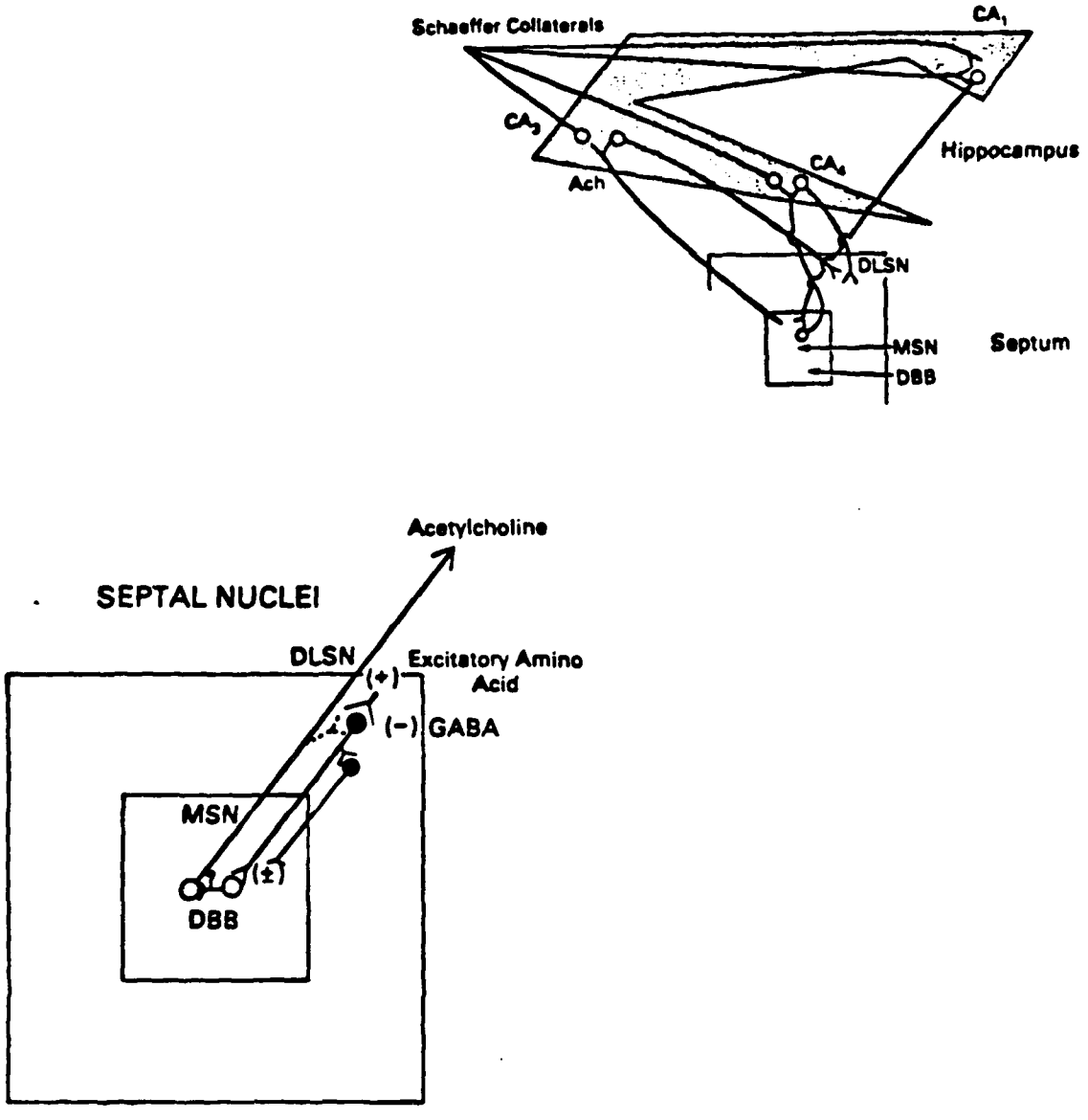

Fig. 1. Schematic diagram comparing hippocampal-septal circuitry (top) with intrinsic circuitry in the septum (bottom).

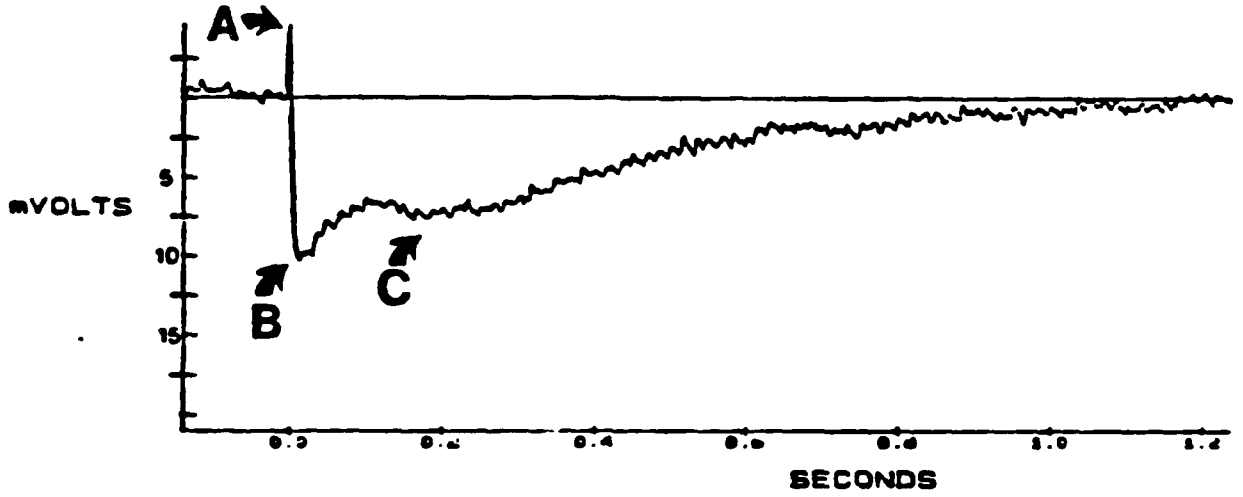

Fig. 2. Typical orthodromic-induced triphasic synaptic responses recorded intracellularly from DLSN neuron. Stimulus is applied focally within the slice and activates fimbrial input to the septum from the hippocampus. A. EPSP mediated by excitatory amino acid acting on quis/kainate receptor. B. Fast ipsp mediated by GABA acting on the $GABA_A$ receptor. C. LHSP thought to be mediated by GABA acting on the $GABA_B$ receptor.

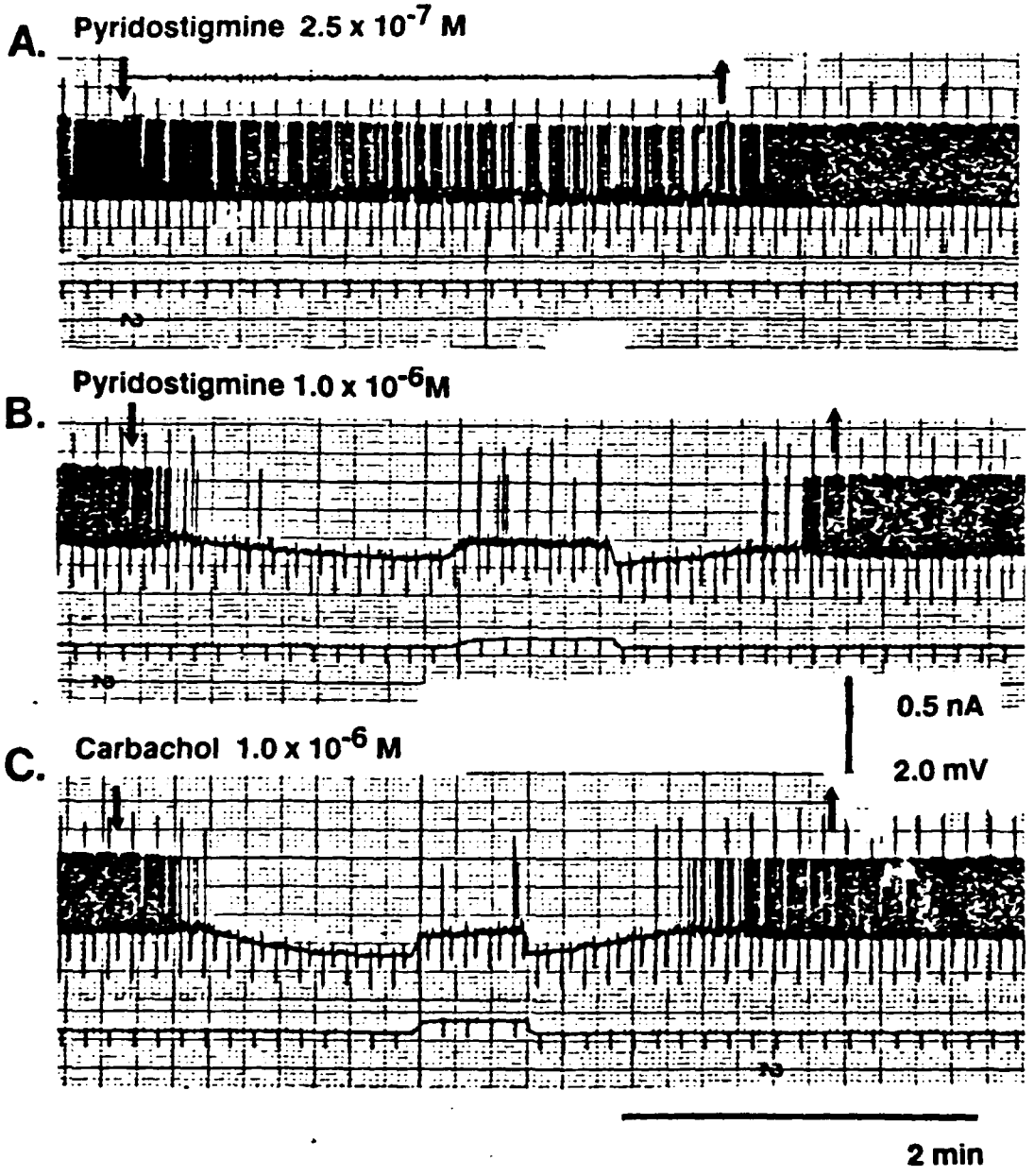

Fig. 3. Effects of pyridostigmine and carbachol on membrane potential, input resistance and spontaneous activity of DLSN neuron. A: Lowest effective concentration of pyridostigmine (2.5×10^{-7} M) to slow spontaneous activity and hyperpolarize the membrane. B: Pyridostigmine (1×10^{-6} M) stops spontaneous firing, hyperpolarizes the membrane and decreases the input resistance of same neuron. C: Carbachol (1×10^{-6} M) produces effects almost identical to those of the equimolar concentration of pyridostigmine.

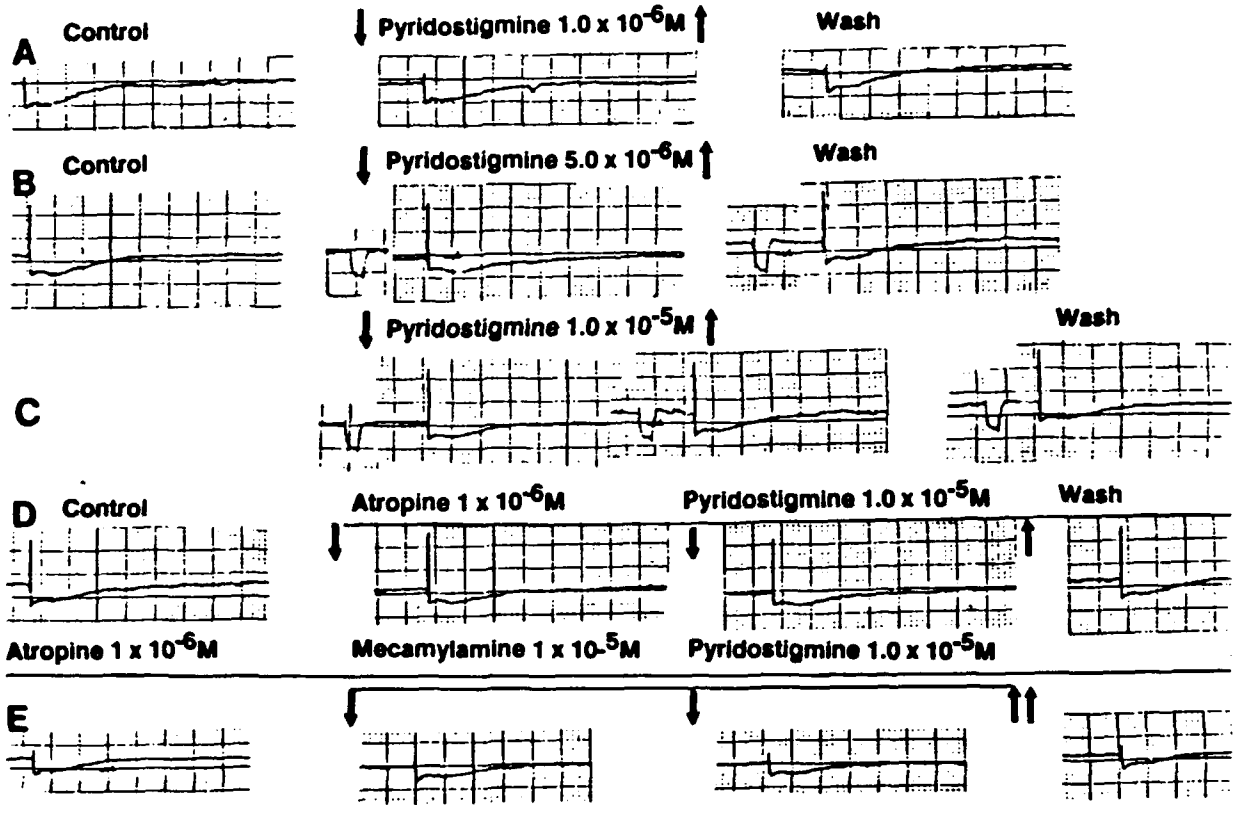

Fig. 4. Effects of pyridostigmine (P) on membrane potential, input resistance and synaptic responses induced orthodromically and recorded from DLSN neuron. A,B, and C. Concentration-dependent effects of pyridostigmine at, 10^{-6}, 5 x 10^{-6} and 10^{-5}M, respectively. Note membrane hyperpolarization and depression of ipsp/LHSP responses. D. Atropine, 10^{-6}M, fails to alter effect of P on membrane potential or inhibitory synaptic responses. E. Addition of mecamylamine, 10^{-5}M, blocks actions of P; i.e., no hyperpolarization or depression of inhibitory synaptic responses is observed.

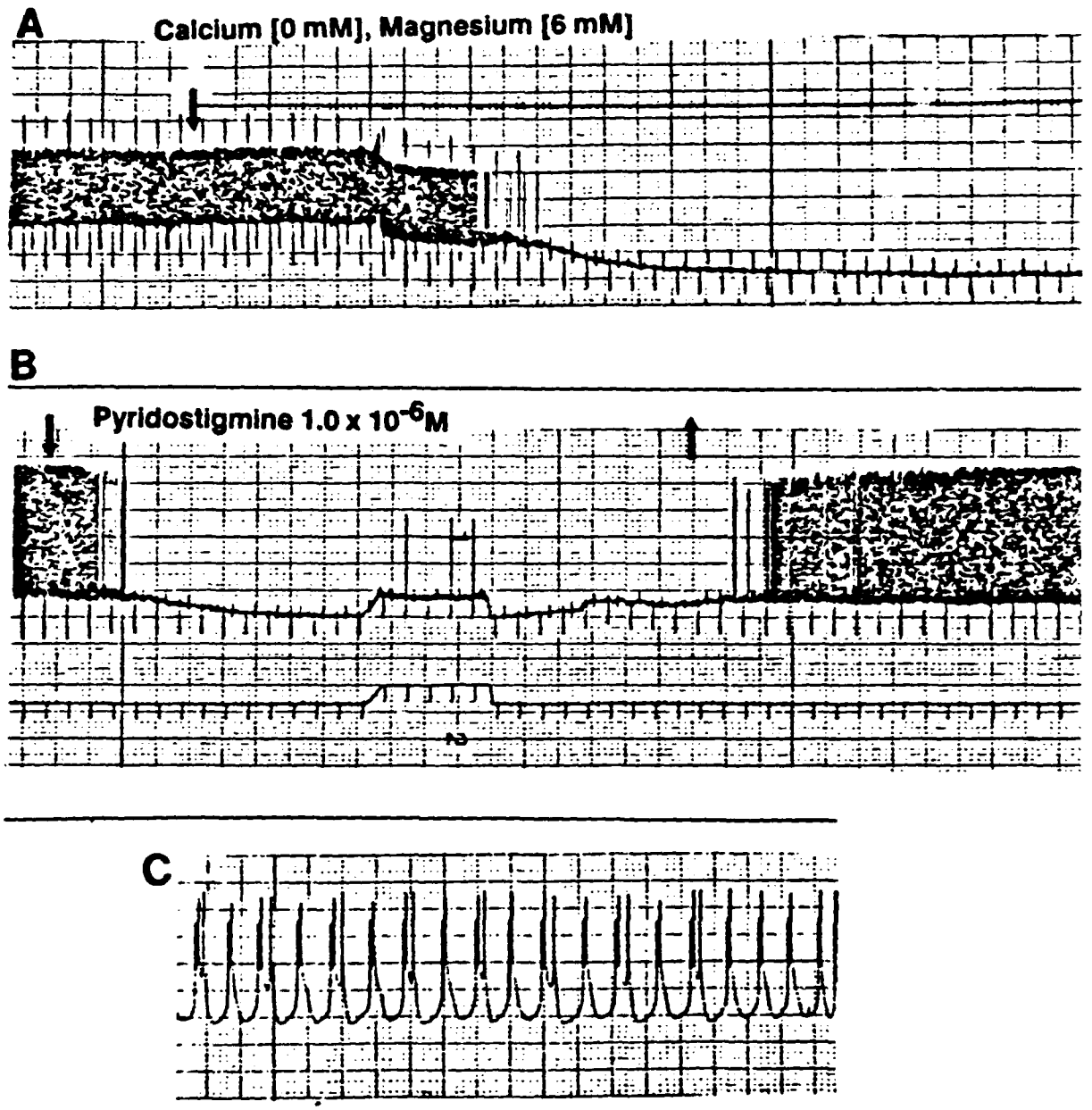

Fig. 5. Pyridostigmine (10^{-6}M) action when synaptic activity is blocked by a zero calcium/high magnesium (6mM) superfusion solution. A: Zero calcium solution blocks spontaneous action potentials and synaptic activity. B: Pyridostigmine is still able to hyperpolarize DLSN neuron and block rhythmic bursting activity (C) which became apparent in zero calcium solution.