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Effects Of Tetrahydroaminoacridine On Nicotinic Acetylcholine Receptors: Studies At Macroscopic And Single-Channel Levels.

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EFFECTS OF TETRAHYDROAMINOACRIDINE ON NICOTINIC ACETYLCHOLINE RECEPTORS: STUDIES AT MACROSCOPIC AND SINGLE-CHANNEL LEVELS

by

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A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Program in Medical Genetics in the Graduate School, The University of Alabama at Birmingham

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ABSTRACT OF DISSERTATION
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Title Effects of Tetrahydroaminoacridine on Nicotinic Acetylcholine Receptors: Studies at Macroscopic and Single-Channel levels

There is a paucity of cholinergic activity in the brains of persons suffering from Alzheimer Disease (AD). Among the therapies for AD is the administration of anticholinesterases such as physostigmine and 1,2,3,4-tetrahydro-9-aminoacridine (THA). These drugs possess chemical moieties similar to those found on the natural neurotransmitter, acetylcholine (ACh). Therefore, these drugs may exert their pharmacological effects not only by inhibiting acetylcholinesterase (AChE) but also by interacting with the nicotinic acetylcholine receptor (nAChR). These studies were designed to demonstrate the effects of physostigmine and THA on the nAChR.

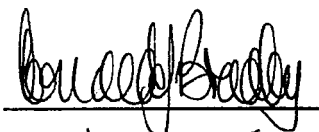
Physostigmine and THA inhibited carbamoylcholine-stimulated inward flux of sodium ions into clonal PC12 cells, a rat pheochromocytoma cell line that expresses ganglionic nAChR. Inhibition occurred with pharmacologic specificity and at physiological concentrations. Inhibition of flux was dose-dependent and onset and recovery were dependent upon the length of incubation.

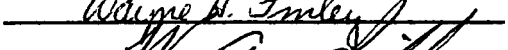
Physostigmine and THA blocked the binding of polyclonal anti-AChR antibodies to nAChR. These drugs not only bound to ligand-specific sites, such as those for bungarotoxin and acetylcholine, but also demonstrated the ability to bind to widely different regions of the receptor.

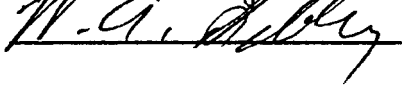
Patch clamp electrophysiological studies were carried out using BC_3H-1 cells, a clonal cell line from mouse neuroblastoma that expresses muscle-type nAChR. The

effects of physostigmine and THA at low concentrations were compared to those of QX-222, a derivative of the local anesthetic, lidocaine, which follows a simple sequential model of open-channel blocking. Physostigmine and THA also blocked channels but deviated from the predictions of the sequential model in that their rates of channel blocking were strongly dependent not only upon membrane voltage but also upon drug concentration. Therefore, channel function was altered by mechanisms in addition to simple blocking.

It was concluded that physostigmine and THA were channel modulators at physiological concentrations. Channel blockade may have occurred by interaction with agonist-binding sites on the receptor or other sites that allosterically interfered with channel function. Blockade may have also occurred by obstruction of the open nAChR channel or by desensitization. Therefore, these drugs paradoxically may oppose their well-established anticholinesterase effects by acting as nAChR antagonists at physiological concentrations.

Abstract Approved by: Committee Chairman 

Program Director 

Date 8/28/92 Dean of Graduate School 

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"If he had only learnt a little less, how infinitely better he might have taught much more!"

-Charles Dickens, Hard Times

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LIST OF ABBREVIATIONS

4-AP	4-Aminopyridine
^{125}I-BGT	^{125}I-Bungarotoxin
A/D	Analog to digital
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AD	Alzheimer Disease
ChAT	Choline acetyltransferase
DAP	3,4-Diaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
EPP	End plate potential
HACU	High affinity choline uptake
HP 029	(±)-9-Amino-1,2,3,4-tetrahydroacridin-1-ol
HSB	High-salt buffer
IgG	Immunoglobulin G
IU	International units
LSB	Low-salt buffer
MG	Myasthenia gravis
nAChR	Nicotinic acetylcholine receptor
NEM	N-ethylmaleimide
NFT	Neurofibrillary tangle

NP	Neuritic plaque
PBS	Phosphate buffered saline
PSP	Post-synaptic potential
TEA	Tetraethylammonium
THA	1,2,3,4-tetrahydro-9-aminoacridine

INTRODUCTION

Increasing age in humans and other mammals correlates with certain changes in gross and microscopic features of the brain (Henderson & Finch, 1989). These changes may ultimately result in a severe dementia first described in detail by Alois Alzheimer (Alzheimer, 1907/1977). Alzheimer's subject was a 51-year-old female who presented, over the course of 4 1/2 years, progressive memory loss, personality changes, language disturbances, and movement disorders. The remarkable feature of Alzheimer's report was the autopsy results wherein he described numerous neurofibrillary tangles affecting cortical neurons that he visualized using the newly developed Bielschowsky silver staining technique. The presenile dementia described by Alzheimer was later determined to be a separate entity in psychiatry. Alzheimer disease (AD) has been shown over the years to occur in persons of widely varying ages, and the pathological and clinical findings have been no more than ambiguous in distinguishing between presenile and senile forms of the disease (Henderson & Finch, 1989). Summarizing a recent epidemiological study has a sobering effect when one realizes the number of persons in the United States alone who may one day be afflicted with Alzheimer disease (Evans, Funkenstein, Albert, Scherr, Cook, Chown, et al., 1989). The authors concluded that of persons over the age of 65 years, 65 to 74 years, 75 to 85 years and those over 85 years, 10.3%, 3%, 18.7% and 47.2%, respectively, had probable AD. It is thus likely that AD is not an uncommon disease and the future increase in population will challenge us to respond to the demanding and expensive health care needs of a large population of demented, helpless victims of Alzheimer disease (Bartus, 1989).

Alzheimer Disease Pathology

As defined clinically, AD is a progressive dementia of insidious onset and is progressively deteriorating (Perl & Pendlebury, 1987). Onset of AD is associated with memory dysfunction, dysphasia, and depression. As the disease progresses, victims become demented, incontinent, and unable to care for themselves although their motor skills remain essentially intact (Damasio, 1992). There is considerable overlap between AD and other age-associated dementing illnesses, among them are Parkinson's disease (Boller, Mizutani, Roessmann, & Gambetti, 1980), multi-infarct dementia (Ojeda, Mastaglia, & Kakulas, 1986), and the viral-induced dementias, Pick's and Creutzfeld-Jakob disease (Watson, 1979; Ball, 1980). Therefore, strict diagnosis is made histopathologically, post-mortem (Wisniewski, Rabe, Zigman, & Silverman, 1989).

Microscopic changes in the AD brain include regionalized loss of neurons and the abundant presence of neurofibrillary tangles (NFT) and neuritic plaques (NP)(Henderson & Finch, 1989). Neuritic plaques (NP) are large, 50 to 200 μm structures found throughout the neocortex. They are formed by clusters of degenerating dendrites and axon terminals surrounding a dense core of amyloid. There is a strong positive correlation between cognitive impairment and the number of NP and NFT found in the brains of AD patients (Blessed, Tomlinson, & Roth, 1968). Neurofibrillary tangles are degenerated neuronal soma, the cytoplasm of which is filled with aggregates of double-stranded, helical filaments known as paired helical filaments (PHF), first described nearly 30 years ago (Kidd, 1963, 1964). These abnormal aggregates arise in cells of particular regions of the neocortex. Most prominently affected are the hippocampus, amygdala, hypothalamus, and the nucleus basalis of Meynert (Whitehouse, Price, Clark, Coyle, & DeLong, 1981; Whitehouse, Price, Clark, Coyle, & DeLong, 1982; Whitehouse, Parhad, Hedrenn, Clark, White, Struble, et al., 1983) which may lose up to 90% of its neuronal components. NFT and NT appear in the brains of normal aged individuals but to a far less extent than found

in the brains of AD victims. The extent of these lesions in AD compared to those in normal elderly leaves little overlap and facilitates diagnosis (Ball, 1977).

Biochemical changes in the brains of AD victims stem from the loss of serotoninergic, noradrenergic, and cholinergic neurons (Bowen, Allen, Benton, Goodhardt, Haan, Palmer, et al., 1983; Cross, Crow, Ferrier, Johnson, Bloom, & Corsellis, 1984; Cross, Crow, Johnson, Joseph, Perry, Perry, et al., 1983; Crow, Cross, Cooper, Deakin, Ferrier, Johnson, et al., 1984; Perry, 1987). The deficit in cholinergic markers in AD brains is the most consistent and well-studied phenomenon since the discovery in 1976 of a specific deficit in choline acetyltransferase (ChAT) in autopsy material from AD victims (Davies & Maloney, 1976; Bowen, Smith, White, & Davison, 1976; Perry, Perry, Blessed, & Tomlinson, 1977; White, Goodhardt, Keet, Hiley, Carasco, Williams, et al., 1977). This enzyme catalyzes the formation of acetylcholine from its precursors, choline and acetyl-coenzyme A (Wurtman, Hefti, & Melamed, 1981). Underlying this loss of ChAT is the loss of cholinergic neurons in the nucleus basalis of Meynert which supplies up to 90% of the cholinergic input to the neocortex (Coyle, Price, & DeLong, 1983; Mesulam, Mufson, & Levey, 1983). These observations support the cholinergic hypothesis which proposes that cholinergic neurons in the brain are significantly responsible for memory and cognition.

Learning and memory have been linked to cholinergic systems in the brain by pharmacological investigations of the muscarinic antagonist, scopolamine (Passagli & Pepeu, 1964; Drachman, 1977; Bartus, Dean, & Beer, 1982). The administration of scopolamine may result in a "scopolamine dementia" that is accompanied by deficits in cognition and memory acquisition. The specificity of cholinergic neuronal function in the processes of memory and cognition was evaluated by the administration of scopolamine to normal volunteers (Drachman, 1977). Two drugs were compared for their ability to reverse the amnestic effects of scopolamine: physostigmine, an anticholinesterase, and d-amphetamine, a catecholamine agonist. Because these drugs have specific effects on

particular neuronal systems, antagonism of the effects of scopolamine by one drug or the other would discriminate between the system affected by scopolamine. It was found that the effects of scopolamine were antagonized by physostigmine which offers evidence that cholinergic neurons specifically participate in the processes of memory and cognition. There are similarities between the memory and cognitive deficits in aged humans and the deficits produced by the administration of scopolamine (Drachman & Leavitt, 1974). Therefore, the administration of physostigmine to victims of AD has received much attention and many clinical trials (Becker & Giacobini, 1988). Inhibitors of acetylcholinesterase produce their pharmacologic effects by increasing the effective concentration ACh in cholinergic synapses. In the brains of victims of AD, the most consistently observed phenomenon is a paucity of choline acetyltransferase, the enzyme responsible for the synthesis of ACh (Bowen, et al., 1976; Davies & Maloney, 1976; Perry, et al., 1977; Rinne, Säkö, Paljärvi, Mölsä, & Rinne, 1988). There also exists reductions in brain nicotinic acetylcholine receptors (Kellar, Whitehouse, Martino-Barrows, Marcus, & Price, 1987; Whitehouse, Martino, Marcus, Zweig, Singer, Price, et al., 1988; Chini & Clementi, 1989) without a concomitant reduction in brain muscarinic AChR as determined by [^{3}H]quinuclidinyl benzilate binding studies (Kellar, et al., 1987). The reductions in nicotinic binding site densities in particular areas of the brain lend further support to the post-mortem diagnosis of AD since the loss of [^{3}H]-ACh and [^{3}H]-nicotine are closely correlated to the disease process (London, Ball, & Waller, 1989). Therefore, the rationale for anticholinesterase therapies in AD is that increasing ACh concentrations directly or indirectly at affected cholinergic synapses in the brain may compensate for the losses of the machinery necessary for proper neuronal function.

The most accessible explanation for the effects of anticholinesterase therapies in the treatment of dementia is simply the inhibition of acetylcholinesterase and the subsequent increase in synaptic ACh concentration. However, there is a sizeable body of literature suggesting that these drugs may influence synaptic transmission by mechanisms that are

distinct from those involving the inhibition of the AChE enzyme. Acetylcholine reacts with high affinity with at least two distinct proteins within the synapse; the nAChR and AChE. It may therefore be expected that the drugs which inhibit AChE may possess chemical groups that are similar or identical to those found on acetylcholine. It follows that these drugs may also interact with not only AChE but the nAChR as well. There are many studies that indicate anticholinesterases directly influence the nAChR at physiological concentrations. AChE inhibitors are effective at very low concentrations (Figure 1). The serum concentrations of anticholinesterases administered therapeutically reach levels far beyond those necessary for enzyme inhibition (Seifert & Eldefrawi, 1974).

Therapeutic Avenues in AD

The biochemical changes observed in AD brains suggest strategies for treatment that logically begin with improving or restoring function to cholinergic systems. There have been four main approaches used in the treatment of Alzheimer disease: 1) nicotinic and muscarinic agonists have been administered, 2) ACh precursor loading with choline, 3) AChE inhibition, and 4) combining precursor loading with AChE inhibition.

Muscarinic agonists have been administered but with mixed results. These agents are toxic and thus side-effects pose serious limitations to clinical trials with these drugs. Oxotremorine was found to produce depression in AD patients (Davis, Hollander, Davidson, Davis, Mohs, & Horvath, 1987). Pilocarpine similarly produced no positive effects (Caine, 1980). Bethanecol reportedly resulted in some improvement in AD patients when it was given intraventricularly (Harbaugh, Roberts, Coombs, Saunders, & Reeder, 1984) although repeats of this study produced equivocal results (Read, 1988; Penn, Martin, Wilson, Fox, & Savoy, 1988). However, a receptor in the continued presence of its agonist will desensitize for a prolonged period. During this time no transmission may be possible and the basis for agonist administration may therefore be faulty.

It has been demonstrated that supplemental dietary choline prevents brain ACh depletion in the presence of antimuscarinic agents (Wecker, Dettbarn, & Schmidt, 1978),

but precursor loading in the form of choline or dietary lecithin have been almost uniformly negative (Becker & Giacobini, 1988). Moreover, iontophoresis studies failed to show that extracellular choline augments cholinergic transmission in the presence of physostigmine (Krnjevick & Reinhardt, 1980). Choline is itself a cholinergic agonist and as such is capable of producing considerable receptor desensitization (Sterz, Peper, Simon, Ebert, Edge, & Bradley, 1986). Since it is not possible to enhance cholinergic activity by providing more precursor, it is doubtful that these methods will ever yield reproducible, positive results.

Presynaptic methods of increasing the amount of neurotransmitter released during synaptic functioning have been tested in vitro using potassium channel blockers, 4-aminopyridine (4-AP), 3,4-diaminopyridine (DAP), and tetraethylammonium (TEA). Block of potassium channels prolongs the depolarization of the presynaptic membrane which activates the voltage-sensitive calcium channels involved in excitation-secretion coupling and thus facilitates the release of neurotransmitter. However, the administration of 4-AP may not be efficacious because of the high concentrations that would be necessary to affect transmitter release and the possible effects on non-cholinergic systems. Drukarch et al. (Drukarch, Kits, Leysen, Schepens, & Stoof, 1989) found that TEA and 4-AP enhanced electrically-evoked and non-evoked release of [^{3}H]-ACh from preparations of rat striatum. These effects were specific to cholinergic neurons. The minimal concentrations of TEA and 4-AP needed to observe significant enhancement of electrically evoked transmitter release were 30 μM and 3 μM, respectively. Enhancement of ACh release from rat striatal synaptosomes by 4-AP was also observed by Buyukuysal and Wurtman (Buyukuysal & Wurtman, 1989). They compared the effects of 1,2,3,4-tetrahydro-9-aminoacridine (THA), a cholinesterase inhibitor, on the high-affinity choline uptake (HACU) and release of newly synthesized acetylcholine. It was found that 50 μM or greater THA blocked HACU and suppressed the release of newly synthesized ACh while 4-AP, at concentrations of 5 μM and greater, stimulated HACU and enhanced the release

of acetylcholine. If a potent channel blocker of this type could be found without side-effects it might be useful in AD, but studies at the neuromuscular junction indicate that such molecules are not as effective as AChE inhibitors in reversing synaptic block (Bradley, Edge, Moran, & Freeman, 1988).

Inhibitors of AChE have been administered alone and in combination with ACh precursors in therapeutic studies for Alzheimer disease. The rationale for administering anticholinesterases is that by inhibiting the enzyme responsible for the breakdown of ACh, the effective concentration of neurotransmitter in the synapse is raised and thus the probability of successful synaptic transmission is enhanced. The use of anticholinesterases to improve synaptic transmission is already common in the treatment of myasthenia gravis (MG). In MG, there is a loss of postsynaptic nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction due to an autoimmune attack. The end-plate potential is then sometimes too small to produce an action potential. By inhibiting AChE, the effective concentration of ACh reaching postsynaptic receptors after presynaptic release is increased, the probability of receptor activation increases, and the end-plate potentials are subsequently increased in amplitude and decay-time so that the potential threshold resulting in an action potential is reached. The anticholinesterases used in the treatment of MG are quaternary amines and thus do not cross the blood-brain barrier into the brain. Uncharged molecules must be used if they are to have effects in the central nervous system. Three such anticholinesterases are currently being used experimentally in AD patients: physostigmine, tetrahydroaminoacridine (THA), and HP 029, a congener of THA. There is no evidence that physostigmine administered concomitantly with ACh precursors produce improvements in AD patients in a more consistent fashion than physostigmine alone (Becker & Giacobini, 1988). There have been many studies on the effects of physostigmine on memory and cognition (Drachman, 1977; Becker & Giacobini, 1988). Some studies have noted improvements in cognitive functioning (Becker & Giacobini,

1988). The use of physostigmine is limited because of its short half-life of about 30 min in the blood or brain (Sharpless & Thal, 1985).

Analogs of physostigmine have been prepared that have improved stability, which results in slower reversibility of AChE inhibition, and other characteristics that may enhance their ability to cross the blood-brain barrier (Marta, Castellano, Pavone, Pagella, Brufani, & Pomponi, 1988). Physostigmine analogs with longer N-carbamic chains were synthesized and characterized in vitro and in vivo. The reactivation constants were evaluated on AChE extracted from electric fish, and several compounds tested exhibited reversal constants that were 7 to 11 times higher than physostigmine ($t_{rev} \approx 20$ min). Although doses administered were up to 150 times that of physostigmine (0.03 mg/kg), the LD_{50} for physostigmine (≈ 0.6 mg/kg) is also very much lower. Hence, these analogs may prove to be attractive alternatives to physostigmine or even THA.

The use of THA in the treatment of AD was first reported by Summers, Viesselman, Marsh, and Candelora (1981). In their study, 75% of AD patients showed memory and cognitive improvements after acute intravenous injections of THA. These authors published a subsequent report on the long-term effects of oral-dosed THA therapy and found improvement in 16 out of 17 patients in the study (Summers, Majorski, Marsh, Tachiki, & Kling, 1986). Other more recent studies have partially reproduced the effects observed by Summers (Gauthier, Masson, Gauthier, Bouchard, Collier, Bacher, et al., 1988; Nyback, Nyman, Ohman, Nordgren, & Lindstrom, 1988; Kumar, Murphy, Sherman, Ashford, & Markwell, 1988). Compared to physostigmine, THA has been found to be 50-fold less potent but its effects persisted twice as long (Hunter, Murray, Jones, Cross, & Green, 1989). A drawback in the use of THA is its hepatotoxicity, although its toxic effects have been reported to be controllable by dosage regulation and even to be reversible (Summers, 1989). The 1-hydroxy derivative of THA (HP 029) has been studied in rats and is less hepatotoxic (Shutske, Pierrat, Cornfeldt, Szewczak, Huger, Bores, et al., 1988).

Physiological Considerations of the Actions of Anticholinesterases. The potential use of THA as a therapy in AD has stimulated much interest and has therefore spawned many studies of its electrophysiological and biochemical effects. It was determined that THA is potent inhibitor of rat brain AChE with an IC_{50} of about 57 nanomolar (Hunter, et al., 1989). This is so low that its effects may simply be mediated by its properties as an anticholinesterase. Other studies indicate that it may influence ionic channels and exert dual or even multiple effects independently of its anticholinesterase effects (Perry, Smith, Court, Bonham, Rodway, & Atack, 1988a; Stevens & Cotman, 1987). It has been found to inhibit potassium conductances at many-fold the clinical concentrations in rat hippocampal slices (Stevens & Cotman, 1987; Rogawski, 1987) and ventricular myocytes (Osterrieder, 1987), and it will block potassium channels and slow the inactivation of sodium channels in Myxicola giant axons (Schauf & Sattin, 1987).

Anticholinesterase actions on nerve terminals have been studied, primarily because of the accessibility of the preparations. Nevertheless, direct actions of these drugs on ion channels located on peripheral nerves may be indicative of their central actions. Physostigmine and THA were found to increase cholinergic transmission at mouse neuromuscular junction preparations (Harvey & Rowan, 1988). The authors concluded that these drugs reduced the activity of presynaptic K^+ channels which resulted in enhanced transmitter release, but the concentrations required were far above the concentrations necessary to inhibit the activity of junctional AChE and certainly above clinically relevant concentrations (15 μM - 100 μM). Recently, Elinder, Mohammed, Winblad, and Århem (1989) demonstrated that THA may have promiscuous effects and influence sodium and calcium channels as well as potassium channels. These authors used voltage-clamp experiments with the myelinated nerve fiber of Xenopus laevis. They showed that even 10 μM THA reduced sodium currents by 60% and reduced potassium currents only by about 30%. Thus, the effects of THA may be more significant on sodium channels than on potassium channels, suggesting that the therapeutic effects of THA may

have little to do with its action on potassium channels. The concentrations of THA in these experiments were higher than the serum concentrations found in patients undergoing anticholinesterase therapy, about 297 nM (Park, Tachiki, & Summers, 1986). A recent study in mice demonstrated THA concentrations 10-fold higher in the brain than in the serum (Liston, Russo, Mena, & Williams, 1988). Thus, clinically relevant concentrations may reach micromolar levels.

Most of the studies of the effects of anticholinesterases on tissue preparations have demonstrated their ability to influence ionic channel behavior. It is notable that very few studies have been performed to investigate, at the single channel level, possible direct effects on cholinergic receptors (Pascuzzo, Akaike, Maleque, Shaw, Aronstam, Rickett, et al., 1984; Shaw, Aracava, Akaike, Daly, Rickett, & Albuquerque, 1985; Aracava, Deshpande, Rickett, Brossi, Schönenberger, & Albuquerque, 1987). Potter, Ferrendelli, Hanchett, Hollifield, and Lorenzi (1989) showed that low concentrations of THA inhibited the binding of muscarinic ligands to hippocampal membranes. Evidence that THA and physostigmine block carbachol-stimulated sodium flux in PC12 cells has been presented (Edge, Kemp, & Bradley, 1988). The effects of pyridostigmine, an anticholinesterase possessing a quaternary amine, were studied at micromolar concentrations by Akaike, Ikeda, Brookes, Pascuzzo, Rickett, and Albuquerque (1984) using single-channel methods on rat myoball preparations. Pyridostigmine was found to lower single channel conductance, decrease the frequency of channel opening, produce brief closures within the opening of the channel, and produce bursting activity of channel openings. The authors hypothesized that pyridostigmine induced an altered, desensitized form of the nicotinic acetylcholine receptor. Pyridostigmine, as well as neostigmine, has been shown to inhibit agonist binding to nAChR (Pascuzzo, et al., 1984; Sherby, Eldefrawi, Albuquerque, & Eldefrawi, 1985). Pyridostigmine has also been shown to possess agonist-like properties using iontophoretic application at rat muscle end-plates (Bradley, Sterz, & Peper, 1986b). This may not be surprising since anticholinesterases possess chemical moeities similar to

those of acetylcholine and compete with acetylcholine at the active site on AChE. Studies using this logic in order to examine the effects of anticholinesterases on nAChR have been done for some time (Seifert & Eldefrawi, 1974; Bakry, Eldefrawi, Eldefrawi, & Riker, 1982) although efforts with single-channel methods are relatively new.

Anticholinesterases have been frequently used to reverse neuromuscular blockade after anesthesia (Taylor, 1985). As noted by Wachtel (1990), pyridostigmine, edrophonium, and neostigmine are used interchangeably to reverse blockade even though their individual effects may be dissimilar. Since these drugs have similar pharmacokinetic characteristics, their differing clinical properties may be due to influences on other molecules at the neuromuscular junction. Indeed, using patch clamp methods, Wachtel found that the anticholinesterases noted above reduced the mean channel open time of nAChR in mouse BC_3H-1 cells at clinically relevant concentrations. Her study did not include the burst time behavior of patches containing anticholinesterases, although she noted that it is the total open time of a channel per burst that determines the amplitude of the postsynaptic endplate potential. Anticholinesterases therefore may alter endplate kinetics by acting as channel blockers as well as by inhibiting AChE.

The effects of two anticholinesterases, THA and physostigmine, on the behavior of single channel kinetics as recorded from BC_3H-1 cells were investigated. These effects were compared to the channel blocking behavior exhibited by a positively charged lidocaine derivative, QX-222. It was found that THA and physostigmine blocked the ion channel of the nAChR after it was opened by acetylcholine. This blocking effect was similar to the effects of QX-222 which can be described by a simple linear sequential blocking model. Most of the predictions of the model could be satisified by the effects of THA and physostigmine. However, physostigmine and THA had very potent effects on single channel activity, and they had an additional effect not described by the model which may have indicated that they accelerated desensitization of the receptors.

OBJECTIVES

The use of anticholinesterases in the experimental treatment of AD is based upon the rationale that increasing the amount of ACh at cholinergic synapses in the brain will facilitate neural transmission in areas of cholinergic decline. If inhibition of AChE is the only important aspect of this treatment, then any cholinesterase inhibitor that crosses the blood-brain barrier could theoretically be used therapeutically. However, only THA and physostigmine have been extensively studied to date with claims of success in the treatment of AD dementia. Other treatment modalities aimed at increasing ACh concentrations by presynaptic means have met with no success. It is hypothesized in this study that THA and physostigmine may act upon the nAChR in a way that influences their palliative effects in the treatment of AD dementia.

It is known already that anticholinesterases, including physostigmine and THA, interact with the nAChR but at concentrations greater than those at which most AChE would be inhibited. However, the physiological concentrations of these drugs may be within a range great enough to allow appreciable interaction with nAChR. It should be possible to determine the effects of physiological concentrations of anticholinesterases upon receptor preparations in vitro using sensitive techniques such as patch clamp electrophysiology and radioactive tracer fluxes.

Pharmacological differences between THA and physostigmine may extend beyond ACh binding sites and involve binding sites on other areas of the nAChR permitting allosteric effects on the receptor channel. Competition binding experiments between anticholinesterases and ^{125}I-bungarotoxin (^{125}I-BGT) are useful in determining the relative affinities of these drugs for nAChR. However, if other sites on the receptor are involved, they may be demonstrated by immunological methods. It should be possible to

demonstrate these sites by the use of polyclonal antibodies against the nAChR as found in the serum of patients suffering from the neuromuscular disease, myasthenia gravis (MG). Competitive binding between anticholinesterase and antibody should result in decreased antibody binding as measured in conventional assays for anti-AChR antibody.

Measurements of anticholinesterase effects on nAChR behavior can be demonstrated by radioactive tracer ion influx studies. Previous studies using ligand binding inhibition and neurotransmitter release measurement techniques have indicated that anticholinesterases directly block the nAChR but at concentrations that were well above those estimated to be therapeutic (Shaw, et al., 1985; Sherby, et al., 1985; Nilsson, Adem, Hardy, Winblad, & Nordberg, 1987). Ion flux studies may prove to be a sensitive indicator of the types of interactions between anticholinesterases and nAChR (Edge, et al., 1988). It may be speculated that anticholinesterases either block directly by channel blockade or indirectly by desensitization (Aracava, et al., 1987).

Direct measurements of the effects of anticholinesterases on channel behavior can be made using patch clamp electrophysiological techniques. The electrical behavior of individual channels can be recorded and analyzed for open and closed durations, current amplitudes, and total current passed. The patch clamp technique may be the most sensitive method by which to demonstrate how anticholinesterases affect channel behavior and to determine the range of concentrations necessary to bring about significant changes in that behavior. If these drugs act as simple channel blockers, then they will behave according to a simple linear channel blocking scheme that previously has been demonstrated for the anesthetic, QX-222 (Neher & Steinbach, 1978). Otherwise, they may be assumed to act at different locations to bring about blockade through desensitization of the receptor or by competition with agonist at agonist binding sites or even a combination of these.

This dissertation describes a series of studies undertaken to answer the following questions concerning THA and physostigmine and their relationship to the nAChR: (1) At what concentrations do THA and physostigmine inhibit ^{125}I-BGT binding to nAChR? (2)

Do THA and physostigmine interact with sites on the nAChR other than at the primary ligand-binding sites? (3) What are the characteristics of the effects of THA and physostigmine on carbamoylcholine-stimulated tracer ion influx and might they indicate effects other than simple channel blockade? (4) What are the effects of THA and physostigmine on single channel behavior as measured by patch clamp methods? (5) What are the concentration ranges of THA and physostigmine in which significant changes in nAChR activity occur?

MATERIALS AND METHODS

Cell Culture Procedures

The established cell lines, TE671, derived from human medulloblastoma (McCallister, Isaacs, Rongey, Peer, Au, Sonkup, et al., 1977), and BC_3H-1, derived from mouse brain neuroblastoma (Schubert, Harris, Devine, & Heinemann, 1974), were obtained from American Type Culture Collection. PC12 cells, derived from a rat pheochromocytoma (Greene & Tishler, 1976), were obtained from Dr. Ronald Lukas (Barrow Institute, Phoenix AZ). Cells were maintained in proliferative growth phase in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine in 75 cm^2 polystyrene culture flasks kept in a humidified atmosphere of 5% CO_2 in air, at 37 °C. Passages of TE-671 and BC_3H-1 cells were performed at 3 to 6 day intervals. Cells were harvested from confluent flasks following treatment with 0.25% trypsin in sterile Dulbecco's phosphate buffered saline (PBS, Gibco; Grand Island, NY). Trypsin was removed by repeated resuspension in fresh PBS and cold centrifugation at 100 x g. Cells were re-seeded at 1:20 dilution. The PC12 cell line was maintained under the same conditions. Trypsinization was unnecessary since these cells do not readily adhere to the flasks. However, in order to loosen the cells from the plastic, a few ml of cold (4 °C) DMEM were added to facilitate their removal. Because of rapid growth, these cells were split at ratios between 1:25 and 1:40 after passaging.

BC_3H-1 cells for patch clamp experiments were plated at low density onto sterile 22 x 22 mm glass cover slips in 100 mm plastic dishes and maintained in a stringent medium of DMEM containing 0.5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml

streptomycin, and 2 mM L-glutamine. Cells were used in experiments between 4 and 20 days after plating.

nAChR Extraction Procedures

Buffer Preparations. Double-distilled Triton X-100 detergent (ICN Biochemicals; Irvine, CA) was used to solubilize nAChR from human foot muscle or TE671 cells. A working buffer of 0.1% Triton X-100 consisted of 1 mM EDTA·2Na and 5 mM sodium phosphate dibasic/monobasic (24:1), pH 7.40 at 4 °C. An extraction buffer of 2.0% Triton X-100 consisted of 1 mM EDTA·2Na, 1 mM EGTA, and 5 mM sodium phosphate dibasic/monobasic (49:1), pH 7.40 at 4 °C. A Tritonless buffer used for homogenization consisted of 50 mM sodium phosphate dibasic/monobasic (24:1) and 1 mM EDTA·2Na, pH 7.40 at 4 °C.

Extraction Procedures. Human foot muscle from amputated limbs was obtained through Tissue Procurement, Department of Pathology, The University of Alabama at Birmingham. Muscle was separated from tendon and cartilaginous tissues by scraping it with a scalpel on a cold plate (Fisher Scientific; Atlanta, GA). The minced muscle was placed into a Waring blender at 4 °C with cold tritonless buffer at a volume to weight ratio of 4 ml/g tissue. The homogenate was centrifuged in plastic centrifuge tubes of known weight at 29,000 x g for 45 min at 4 °C after which the supernatants were discarded. The pellets were weighed and resuspended in 2.0% Triton X-100 buffer by trituration through a 14 gauge needle at a volume to weight ratio of 0.95 ml/g tissue. Extraction proceeded on ice at 0 °C for 1.5 hr with frequent stirring. The suspensions were then centrifuged at 29,000 x g for 45 min at 4 °C after which the supernatants were carefully withdrawn and pooled. Pooled supernatant was divided into 1 ml aliquots for storage at -70 °C.

Extraction of nAChR from TE671 cells proceded similarly to that for human foot muscle with a few exceptions. Cells were exposed to a few ml of ice-cold DMEM and then scraped from their flasks. Ice-cold Dulbecco's PBS was used to rinse the cells from the flasks into 50 ml plastic centrifuge tubes of known weight. The cells were pelleted by

centrifugation in a table-top centrifuge at 600 x g and at 4 °C. The supernatants were discarded and the pellets were weighed. The cells were resuspended and lysed using 2.0% Triton X-100 extraction buffer in a volume to weight ratio of 1.5 to 1 ml/g tissue. Extraction proceded on ice at 0 °C for 0.5 hr with frequent stirring. The suspensions were then centrifuged at 29,000 x g for 30 min at 4 °C after which the supernatatnts were withdrawn and pooled. Pooled supernatant was divided into 1 ml aliquots for storage at -70 °C.

Receptor Assay. The concentration of nAChR in muscle and cell extractions was determined using radiolabelled bungarotoxin (BGT), a snake toxin from the cobra Bungarus multicinctus that binds specifically and practically irreversibly to the nAChR. ^{125}I-BGT (New England Nuclear; Wilmington, DE) was obtained in lyophilized form. The dry toxin was reconstituted in 1 ml deionized water. Small portions were removed periodically. Before use, the portions were cleaned of debris, unbound toxin, and unreacted radiolabel by loading onto a 2 x 0.5 cm column of Sephadex CM-50 (Pharmacia; Piscataway, New Jersey) in a 5 inch pasteur pipette. The loading volume was 5 ml in 0.1% Triton X-100 buffer. The column was further washed with 15 ml of 0.1% Triton X-100 buffer. Radiolabelled BGT was removed in 100 µl aliquots by adding 0.1% Triton X-100 buffer containing 0.5 M NaCl. Volumes of nAChR extracts ranging from 0.25 to 5.0 µl were incubated with 1 pmol ^{125}I-BGT for 1 hr at room temperature. The total volume of all assays was brought to 200 µl using 0.1% Triton X-100 buffer. Background was determined by first adding an excess of unlabelled BGT (Sigma Chemical Company; St. Louis, MO) to duplicate tubes before adding labelled toxin. Since toxin unbound to nAChR is adsorbed by carboxymethylcellulose, it was removed by passing the mixture over a 2 x 0.5 cm Sephadex CM-50 (Pharmacia) column in a 5 inch pasteur pipette and washing the column with 800 µl 0.1% Triton X-100 buffer. The activity of the toxin-receptor complexes were then determined by counting the washes in a gamma counter.

Anti-AChR Antibody Titer Determination

Complexes of ^{125}I-bungarotoxin-AChR were prepared by incubating Triton X-100 extracts of nAChR from either human foot muscle or TE671 cells with 10-fold excess toxin for 1 hr at room temperature. Unbound toxin was removed by passing the mixture over a 2 x 0.5 cm Sephadex CM-50 column in a 5 inch pasteur pipette and washing the column with an appropriate volume of 0.1% Triton X-100 buffer to remove the receptor-toxin complex and give a specific activity per volume. Dilutions of human MG-patient sera from 0.25 µl to 5.0 µl were incubated with 20 fmoles of toxin-receptor complex overnight brought to a total volume of 200 µl using 0.1% Triton X-100 buffer at 4 °C. Background was determined by using comparable amounts of control sera obtained from persons without neuromuscular disease. The antibody-receptor complexes were precipitated with 20 µl of goat anti-human IgG (Research Products International; Mt. Prospect, IL). The tubes were left to stand at room temperature for 45 min and then at 4 °C for 3 hr. The immune precipitates were collected by centrifugation at 1000 x g. The pellets were washed without resuspension in 800 µl of ice-cold 0.1% Triton X-100 buffer. After centrifugation, the supernatants were discarded and radioactivity in the pellets was measured with a gamma counter, the background counts were subtracted from patient samples, and a titer of anti-AChR in nmoles/L serum was calculated.

Antibody Inhibition Assay

Varying concentrations of anticholinesterases were incubated overnight at 4 °C with 20 fmoles of nAChR obtained from TE671 cells or human foot muscle. Physostigmine was obtained from Sigma Chemical Company, THA from Aldrich (Milwaukee, WI), and ambenonium was generously provided by Sterling Drug Company (New York, NY). Patient sera from known myasthenics with high anti-AChR antibody titers were then added for a final dilution of 1:40 in a total volume of 190 µl and incubated overnight at 4 °C. The final volume was brought to 200 µl with 0.1% Triton X-100 buffer containing ^{125}I-BGT for a final concentration of 1 x 10^{-12} M. After incubating at 4 °C for 3 hr, the complex

was immunoprecipitated by the addition of 20 µl goat anti-human IgG incubated with the complex at 4 °C for 3 hr. The precipitate was collected by centrifugation at 8,000 x g in a refrigerated centrifuge, the supernatant was withdrawn, and the pellet was washed with 800 µl of 0.1% Triton X-100 buffer. The pellet was then counted in a gamma counter. Inhibition was calculated as the concentration of toxin binding sites present at each concentration of drug compared to the concentration of toxin binding sites in the absence of drug.

Toxin Inhibition Assay

Varying concentrations of anticholinesterases were incubated overnight at 4 °C with 5 to 10 fmoles of nAChR obtained from TE671 cells or human foot muscle. The total volume of the assay was brought to 200 µl by the the addition of 10 µl 0.1% Triton X-100 buffer containing 1 pmole ^{125}I-BGT. The mixture was left to stand at 24 °C for 1 hr before passing it over a 2 x 0.5 cm pasteur pipette column of Sephadex CM-50. The column was washed with 800 µl of 0.1% Triton X-100 buffer. The washes contained toxin bound to receptor which was then counted in a gamma counter to obtain the number of toxin binding sites that were present. Controls contained no anticholinesterase and were valued as the amount of labelled toxin bound to receptor minus a background determined by the addition of an excess of unlabelled toxin.

Sodium Influx Procedure

The measurement of sodium influx was performed as published by Kemp and Edge (1987). PC12 cells were plated onto plastic 5 cm culture dishes that had been previously coated with 0.1% poly-L-lysine (Sigma Chemical Company) in deionized water. After a brief period during which the cells became attached to the bottom of the dish, the culture medium was removed and the cells were incubated in HEPES buffer composed of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose, and 25 mM HEPES, adjusted to pH 7.4 with Tris base. Cells were incubated up to 30 min in various concentrations of anticholinesterase drugs. The incubation with drug

was interrupted by replacing the incubation buffer with 1 ml HEPES buffer containing 5 mM oubain, 0.7-1.2 μCi/ml $^{22}Na^+$ (New England Nuclear), and 600 μM carbamoylcholine as agonist to initiate flux. After 20 sec, most of the radioactive solution was aspirated and at 30 sec the dish was quickly immersed into three successive baths of 800 ml ice-cold HEPES buffer to stop the reaction. For some experiments, the drugs under study were not pre-incubated with the cells but were co-incubated with the carbamoylcholine. The cells were then digested in 2 ml of 0.5 M NaOH for 12 hr with vigorous shaking. Radioactivity was measured by counting the digest in a gamma counter. Influx was expressed as the fraction of radioactive tracer found inside the cells per milligram protein per minute flux. Protein concentrations were determined by the method according to Lowry, Rosebrough, Farr, and Randall (1951).

Acetylcholinesterase Purification

Buffer Preparation. Low salt buffer (LSB) consisted of 5 mM N-ethylmaleimide (NEM), and 25 mM Tris base, pH 7.6. High salt buffer (HSB) consisted of 1.0 M LiCl, 50 mM Tris base, 50 mM EDTA•2Na, and 5 mM NEM, pH 7.6. The elution buffer was similar to the HSB except there was no NEM present and 20 mM edrophonium chloride was added. Dialysis buffer consisted of 155 mM NaCl, 10 mM $MgCl_2$, 10 mM EDTA•2Na, and 10 mM Tris base, pH = 7.6.

Affinity Column Preparation. Epoxy-activated Sepharose Cl-6B (Pharmacia) was used to make an edrophonium affinity column to purify AChE in a manner after Hodgson and Chubb (1983). The matrix was swelled and washed per the manufacturer's recommendations. The gel slurry was dried on a Büchner funnel and transferred into a coupling solution containing 0.2 M edrophonium chloride (Sigma), 1 part gel to 2 parts coupling solution. The pH of the solution was adjusted to 12 with 0.5 M sodium hydroxide and shaken for 24 hr at 50 °C. The solution was transferred into a Büchner funnel and washed in sequence with 20 volumes each of 0.1 M sodium acetate buffer, pH 4.5, 0.012 M sodium borate buffer, pH 10.0, and deionized water. The dry cake was

resuspended in 1.0 M ethanolamine and left to stand overnight to block remaining active oxirane groups. The gel was filtered through fritted glass and then washed with 200 ml each of sodium borate buffer, sodium acetate buffer, and water. The Sepharose cake was resuspended in sodium acetate buffer and transferred to a 0.7 x 10 cm Bio-Rad Econo column (Bio-Rad; Melville, NY).

Purification Procedures. Frozen electric organ from the electric fish Narcine braziliensis was extracted, weighed, and homogenized in a cold Eberbach tissue blender in 3 volumes of ice-cold LSB. The homogenate was then centrifuged at 29,000 x g for 35 min. After the second wash in LSB, all pellets were collected into one tube to which was added 25 ml ice-cold HSB. The pellet was resuspended, a stir bar was added, and the homogenate was left to stand at 4 °C on a stir plate for 3.5 hr. After extraction in HSB, the homogenate was centrifuged at 29,000 x g for 1 hr at 4 °C. The supernatant was carefully removed by pipetting and placed into the graduated reservoir of a chromatography column assembly. All subsequent chromatographic and dialysis procedures were performed at 4 °C. AChE extracted into HSB was loaded onto the edrophonium affinity column at 0.75 column volume per hr. After loading, the column was washed with 5 volumes of HSB and then eluted with 29 mM edrophonium chloride elution buffer. Protein in the fraction collected was monitored by a commercially available Bradford protein assay (Bio-Rad). Edrophonium was removed by dialysis against 1,700 volumes of dialysis buffer with frequent changes at 4 °C. Typical AChE yields were 350-400 IU/g starting material, a 1,500-fold concentration. In order to determine immediately after elution which fractions of the eluate contained AChE activity, small aliquots (1.5 ml) of each fraction were desalted on Sephadex G-25M (Pharmacia) fitted into a 50 ml plastic centrifuge tube. This arrangement allowed the column to be centrifuged and, consequently, the void volume could be ignored. The column was equilibrated with 15 ml of deionized water, the void volume was centrifuged out before loading an aliquot to be desalted. Aliquots loaded onto the fritted glass top of the column were forced through the dry column by centrifugation at

1,500 x g for 5 min in a refrigerated, table-top centrifuge and collected in the plastic centrifuge tube surrounding the column.

Determination of AChE Activity. The activity of cholinesterase preparations was determined by a modified method of Ellman, Courtney, Andres, and Featherstone (1961). A stock solution of substrate was made and aliquots were frozen at -20°C until needed. The stock solution consisted of 9 mM acetylthiocholine iodide and 0.7 M 5,5'-dithio-bis-(2-nitrobenzoate) in a buffer composed of 50 mM potassium dihydrogen phosphate and 2 mM magnesium chloride. The buffer pH was adjusted to 7.40 with sodium hydroxide before the addition of substrates. Enzyme preparations were diluted in the phosphate buffer described above with addition of 0.01% gelatin (kerageenan) which significantly increased the lifetime of the enzyme. Enzyme activity assays were performed spectrophotometrically in a 96-well tissue culture plate, and absorbances were measured at 405 nm using a multiscan spectrophotometer (Titertek: Flow Laboratories; McLean, VA). Briefly, 100 µl of enzyme dilution was added to each well. The reaction was initiated by the addition of 100 µl of stock Ellman reagent. The reaction blank consisted of a solution of substrates, enzyme, and the cholinesterase inhibitor, diisopropylfluorophosphate (100 µM). Generally, absorbances were measured every 5 min. After subtraction of the appropriate blank value, absorbances were plotted against time. The value for the slope was used to determine the enzyme activity which was expressed in international units (IU). Activity inhibition assays were performed similarly at a total volume of 200 µl but with the addition of a cholinesterase inhibitor at various concentrations.

Patch Clamp Techniques.

The patch clamp technique (Hamill, Marty, Neher, Sakman, & Sigworth, 1981; Sakman & Neher, 1983) was used to record single channel nAChR currents from the surface membrane of cultured BC3H-1 cells. These cells were maintained under stressful conditions using minimal medium in order to inhibit proliferation and promote differentiation for nAChR expression. For recording, the cells were maintained in a

depolarizing buffer composed of 5.4 mM NaCl, 140 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, 25.0 mM HEPES, and 11.0 mM dextrose, pH = 7.4 at 22 to 24 °C. This buffer was also used to fill the pipettes after having been passed through a teflon 0.2 μm syringe filter (Millipore; Bedford, MA). All experiments used 100 nM ACh as the control. The patch clamp microelectrodes were made from borosilicate capillary glass (Kimax; Fisher Scientific) 1 mm outside diameter. Electrode resistance ranged between 5 and 10 MΩ when filled with recording buffer. Pipettes were manufactured with a Narishige PP-83 vertical pipette puller (Narishige Scientific Instrument Lab; Tokyo, Japan) to a steep taper and an inside tip diameter of 0.5 - 0.8 μm. The tips were coated to within 30 μm with a non-conducting, hydrophobic coating (Sylgard®, Dow Corning; Midland, MI) and then heat-polished on a vertical Narishige MF-83 microforge to reduce the inside tip diameter to 0.3 - 0.4 μm and remove any coating that may have crept over the glass surface and into the tip. In order to estimate the proper tip size before using the pipette, the polished pipette was fitted onto a flexible tube attached to a 10 cc syringe with the plunger withdrawn to 10 cc. The tip of the pipette was lowered into a glass vial of methanol. The plunger was then depressed and at the moment bubbles appeared at the tip of the pipette the volume was read off the syringe. A reading of 4 to 5 cc was considered adequate. The solution bathing the cells and the recording solution was a depolarizing, high potassium buffer that contained no chelating agents. Included in the pipette buffer was 1.0 μM tetrodotoxin (Sigma) to increase the stability of the recordings and improve the signal-to-noise ratio. Micromanipulations were observed on the stage of an Olympus IMT-2 inverted microscope at 800 x through a Hoffmann modulation contrast lens system (Olympus Corporation; Lake Success, NY). The patch pipette was lowered onto the cell surface by a motorized micromanipulator (Model 860A motorizer: Newport Electronics; Fountain Valley, CA) and gentle suction was applied by mouth to form an electrically tight seal between the pipette and the cell surface. After forming a gigaohm seal between the electrode and the cell surface, the pipette was left in place in a cell-attached configuration. The receptors were

then exposed to various drugs contained in the pipette filling solution. A List Electronics LM-EPC-7 patch clamp amplifier (Medical Systems, Incorporated; Great Neck, NY) was used to detect single-channel currents. Pipette holding potentials were selected manually and could be changed via the computer keyboard, the computer interface being capable of passing +10 to -10 DC volts to the patch amplifier. The data were filtered at 3 kHz, digitized via a VR-10A pulse-code modulator (Instrutech Corporation; Elmont, NY), and stored on VHS video tapes for computer analysis at a time after the experiment. All software for patch clamp data acquisition and analysis was obtained from Instrutech Corporation. In order to analyze the data recorded on VHS tapes, they were transferred into a partition of the hard disk of an Atari Mega-ST4 computer (Atari Corporation; Sunnyvale, CA). The data were analyzed using semi-automated programs written for the Atari computer system by Drs. Fred Sigworth and Hubert Affolter at Instrutech Corporation (Colquhoun & Sigworth, 1983).

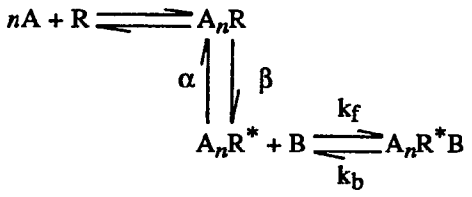
An entire experiment could be stored on VHS video tape. The entire record was not transferred to hard disk for analysis, however. A semi-automated program utilizing an event detection algorithm allowed only portions of the record containing opening events to be transferred to the hard disk for analysis. The program required that a segment of the experiment be played through the computer for a preview of the noise level and event magnitude. The computer screen is imitative of an oscilloscope and allowed viewing 60 ms strips of digitized record at 0.5 Hz. An area of the record with representative noise and no events was selected manually and the average peak-to-peak noise was measured automatically. A multiple of this value was chosen as the threshold above which an event and portions of the immediately surrounding baseline was selected for digital transfer to the hard disk. The threshold value was chosen as one that allowed the detection of as many events as possible without also detecting noise spikes that could contaminate the data to be transferred (Sakman & Neher, 1983). The computer kept track of the time between events even though that portion of the record was not transferred.

Analysis of single channel currents was rigorously restricted to those events which traversed a threshold of half the average peak current value (Figure 7). Although it was obvious some brief events were just missed by this restriction, the use of personal bias to include or reject them would have rendered the data inconsistent. Events shorter than 50 ms were ignored since an accurate determination of their durations and amplitude was not possible due to the cutoff frequency of the filters employed. Records containing more than one channel were analyzed if no more than 0.1% of the open time record contained overlapping events. Overlapping events could then be ignored with no real effect of open time and burst analysis. Classification of burst length was also restricted to the use of a computed value in which the probability of correctly or incorrectly classifying a group of openings as a burst was proportionately equal. Accordingly, a program was written for a microcomputer to evaluate the following equation, the result of which is the minimum closed time between bursts of openings and thus is the maximum closed time between single openings (Colquhoun & Sakman, 1985):

$$\tau_c = e^{-\tau_s/\tau_c} - e^{-\tau_m/\tau_c}$$

The time constants τ_s and τ_m were derived from the slow and intermediate components, respectively, of a maximum likelihood fit of the closed time histogram from each experimental record. This gave the unbiased value τ_c which delineated the closed time between bursts.

Data were analyzed in a manner similar to that described by Neher and Steinbach (1978). The model for analysis is based upon a simple linear sequential blocking scheme:



In this representation of channel events, *A* represents agonist in *n* number per open receptor, *R* is receptor, *B* is blocking drug, and an asterisk denotes an open channel. The

blocked state is represented by A_nR^*B. Various algorithms for kinetic analysis can be derived from this scheme (Neher & Steinbach, 1978). The mean open time of a channel is given by

$$T_o = [\alpha(V) + f(V) \cdot c_B]^{-1} \quad (1)$$

The equation predicts that the mean lifetime of an open channel is the reciprocal of the sum of the rates leading away from the open state. It also implies that the rate of channel blocking, k_f, is dependent upon the concentration of blocking drug, c_B. All rate constants are dependent upon membrane voltage. The mean blocked time of an open channel due to the presence of a channel blocking drug is given by

$$T_b = [k_b(V)]^{-1} \quad (2)$$

Finally, the burst duration should be approximated by the equation

$$T_z = [\alpha(V)]^{-1} \cdot \left(1 + \frac{k_f(V) \cdot c_B}{k_b(V)}\right) \quad (3)$$

The duration of a burst is dependent upon the blocking drug leaving the mouth of an open channel. However, in the linear sequential blocking model, the channel must proceed from the blocked state back through the open state in order to close.

Several predictions of channel behavior in the presence of a blocking drug can be made based on this model and these equations. Equation 1 predicts that channel mean open time must be shortened by the presence of a blocking drug and that mean open time decreases with increasing concentrations of drug. Equation 3 predicts that the mean burst duration is prolonged by increasing drug concentration since the channel in the blocked state can not close directly. The duration of the brief blocking events, or gaps, within a burst is independent of drug concentration. Finally, the amplitude of single channel currents should not be altered by the presence of a channel blocker.

The equations are amenable to solutions of the rate constants by simple graphing and curve fitting methods. The channel openings are random and their durations are exponentially distributed. The known parameters of open time, closed time, and burst

duration can be plotted against the pipette holding potential and the resulting lines fitted to exponential functions by a regression method. The equations of the curve fits provide the information necessary to determine the voltage-dependent rates of channel opening, blocking, and unblocking.

RESULTS

Inhibition of Purified AChE by Various Anticholinesterases

In order to gain a perspective on the relative anticholinesterase potencies of THA and physostigmine, they were compared to other anticholinesterases in a system using AChE purified from the electric organ of an electric fish, Narcine braziliensis. In Figure 1, the IC_{50} values range over 15,000-fold with pyridostigmine being the least potent and ambenonium (AMB) being the most potent inhibitor. Ambenonium is a bisquaternary nitrogen derivative while HP 029 is a 1-hydroxy derivative of THA. HP 029 was a very effective inhibitor but it did not differ significantly from THA. Physostigmine was nearly one-tenth as effective as THA as an anticholinesterase.

^{125}I-BGT Binding Inhibition by Anticholinesterases

Several anticholinesterases showed an ability to inhibit the binding of ^{125}I-BGT to nAChR extracted in Triton X-100 detergent from human foot muscle (Figure 2). The order of potency, decreasing, was AMB > THA > HP 029 > physostigmine. The IC_{50} for these drugs were AMB, 253 μM, THA, 276 μM, and HP 029, 920 μM. Physostigmine did not show any significant inhibition at any concentration used here. The anesthetic QX-222 was also shown to lack any power to inhibit BGT binding even at concentrations as high as 500 μM. This helped to establish that THA interacted with the BGT-binding site whereas physostigmine did not. These results were calculated based on a best-fit linear regression package program (Cricket Graph Software; Philadelphia, PA) for THA, physostigmine, and HP 029. Using the same software, AMB required a logarithmic fit. All regression coefficients were fitted to better than 0.93.

Inhibition of Anti-AChR Antibody Binding to nAChR by Anticholinesterases

Physostigmine could not be shown to inhibit anti-AChR antibody binding to nAChR. However, THA, HP 029, and AMB all inhibited antibody binding at low concentrations. Figure 3 depicts antibodies in the sera of two patients that were blocked by these drugs. Two different patients were used in order to increase the diversity of the antibody population. The drugs exhibited an order of effectiveness that did not reflect the same order of their ability to inhibit the binding of ^{125}I-BGT. Half the antibody binding was blocked by 20 μM HP 029 and 30 μM AMB while THA was much less effective at greater than 150 μM. The low concentrations at which specific blocking occurred was in sharp contrast to the IC_{50} values obtained from toxin inhibition studies. The order of potency of THA and HP 029 is noteworthy since they are totally reversed, presumably by the 1-hydroxy group on HP 029. The decrease in toxin binding sites, therefore, does not reflect a simple inhibition of ^{125}I-BGT binding and indicates that THA, AMB and HP 029 may bind to at least one or more sites outside the ligand binding and toxin binding sites on the nAChR.

Effects of THA and Physostigmine on Ion Flux Into PC12 Cells

The established PC12 tumor cell line was chosen as an accessible system in which to study the effects of THA and physostigmine on ganglionic nicotinic acetylcholine receptors. These studies were simple pharmacological manipulations designed to demonstrate the basic kinetics of inhibition or stimulation of ganglionic nAChR activity by THA and physostigmine. At no concentration up to 5 mM did these drugs stimulate ganglionic receptors. Figures 4 and 5 illustrate the blocking effects of these drugs on carbamoylcholine-stimulated $^{22}Na^+$ influx into PC12 cells. Both drugs exhibited similar pharmacological effects on $^{22}Na^+$ influx. Cells were either preincubated for 30 min with an anticholinesterase or the drug was coincubated with the cells during the flux procedure. The resulting inhibition curves were not significantly different. The IC_{50} values for THA were 17 μM and 18.5 μM for preincubation and coincubation protocols, respectively

Figure 1. Comparison of IC_{50} values of various anticholinesterase drugs. Actual values are: Ambenonium, (AMB) 1.04×10^{-9} M, 1,2,3,4-tetrahydro-9-aminoacridine, (THA) 2.7×10^{-8} M, (±)-9-amino-1,2,3,4-tetrahydroacridin-1-ol, (HP 029) 2.82×10^{-8} M, neostigmine, (NEO) 1.48×10^{-7} M, physostigmine, (PHY) 2.3×10^{-7} M, edrophonium, (EDRO) 1.9×10^{-6} M, pyridostigmine, (PYR) 1.58×10^{-5} M.

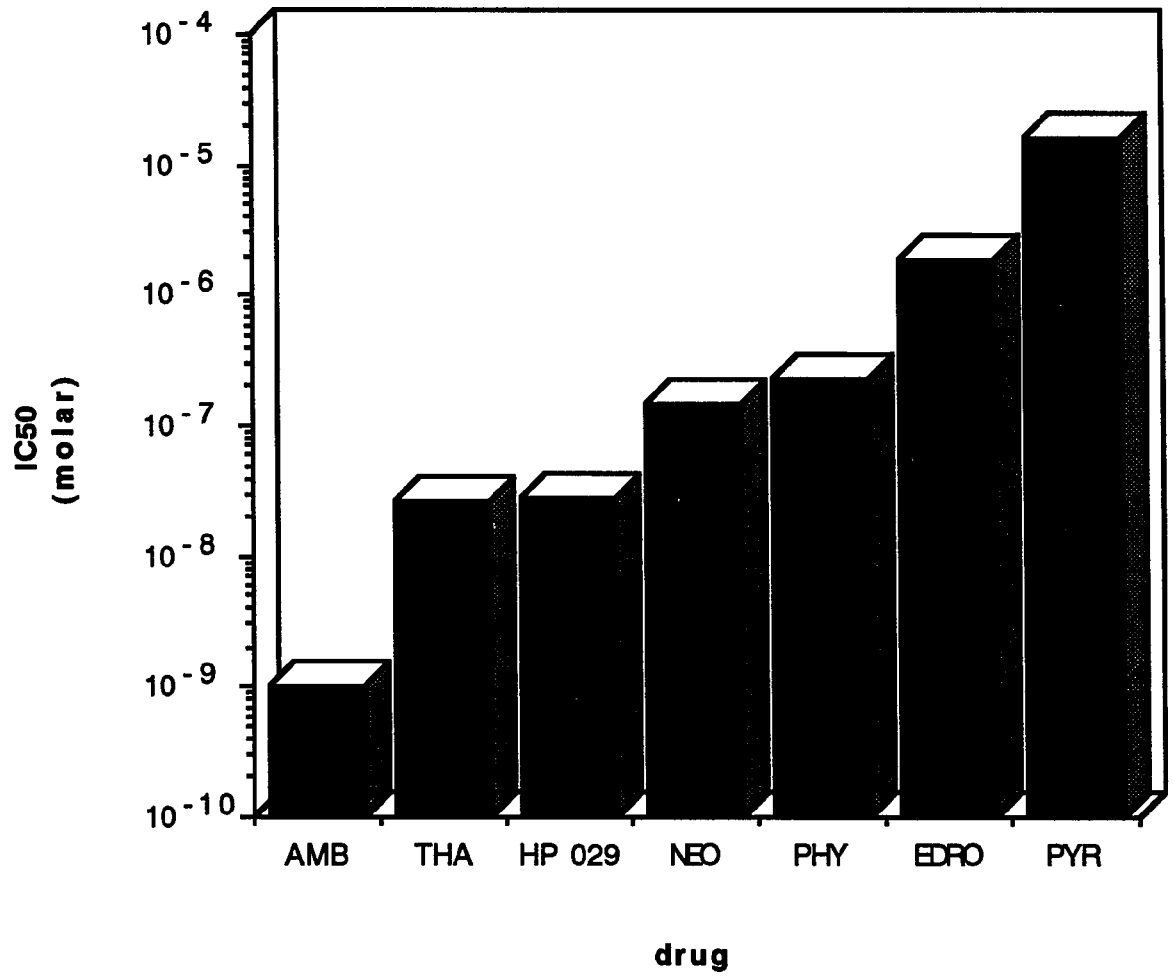
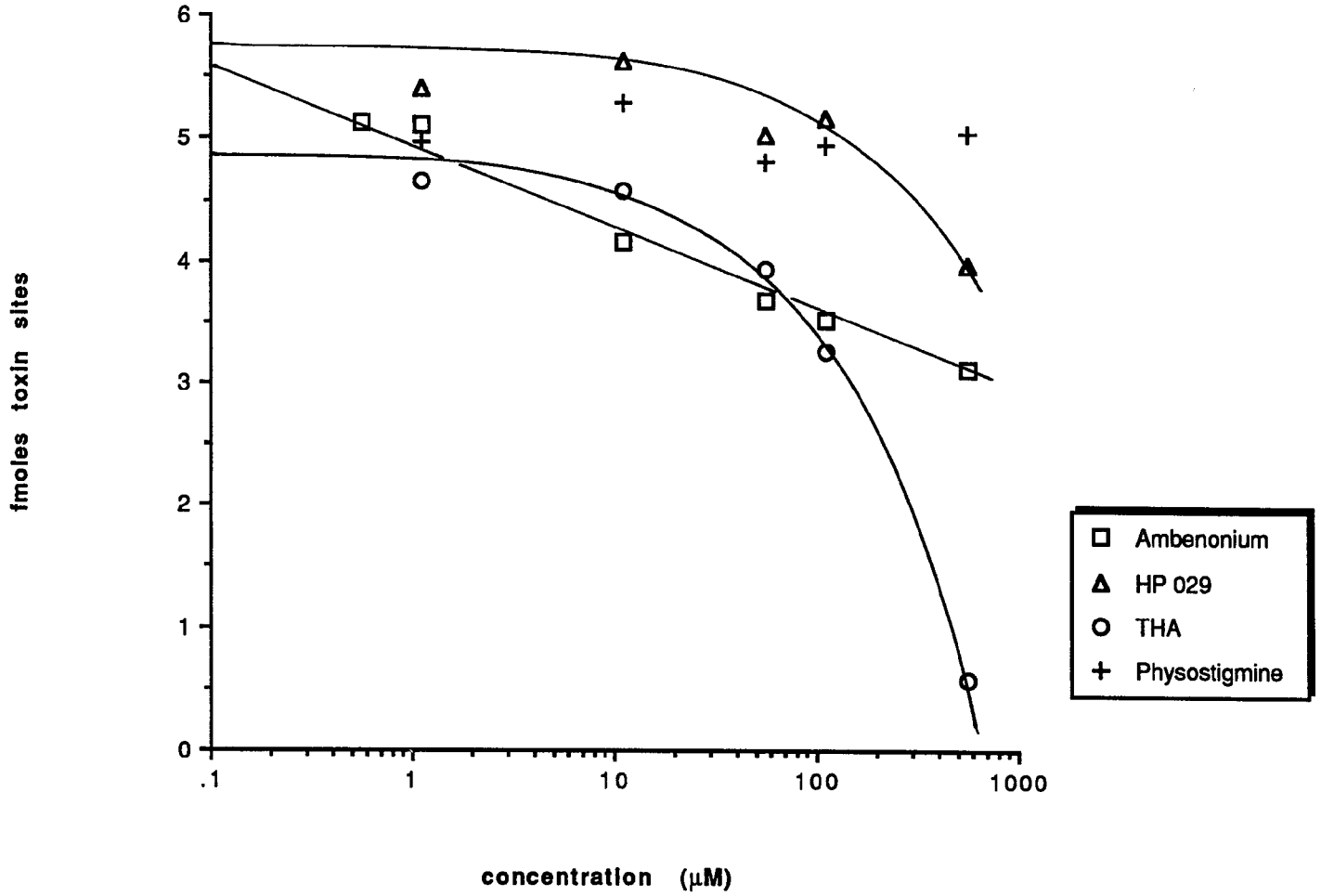

IC50
(molar)
10 - 4
10 - 5
10 - 6
10 - 7
10 - 8
10 - 9
10 - 10
AMB
THA
HP 029
NEO
PHY
EDRO
PYR
drug

Figure 2. Inhibition of ^{125}I-BGT binding to nAChR in the presence of anticholinesterase drugs. Increasing amounts of drugs were preincubated with 6 fmoles receptor from normal human foot muscle before being exposed to ^{125}I-BGT. IC_{50} values were calculated by a best line fit software program and are: AMB, 2.53×10^{-3} M, HP 029, 9.20×10^{-4} M, and THA 2.76×10^{-4} M. Physostigmine showed no inhibition at concentrations used here.


fmoles toxin sites
6
5
4
3
2
1
0
.1
1
10
100
1000
concentration (μM)
Ambenonium
HP 029
THA
Physostigmine

(Figure 4). The IC_{50} values for physostigmine (Figure 5) were similar although somewhat higher at 23.4 μM and 21.7 μM for preincubation and coincubation protocols, respectively.

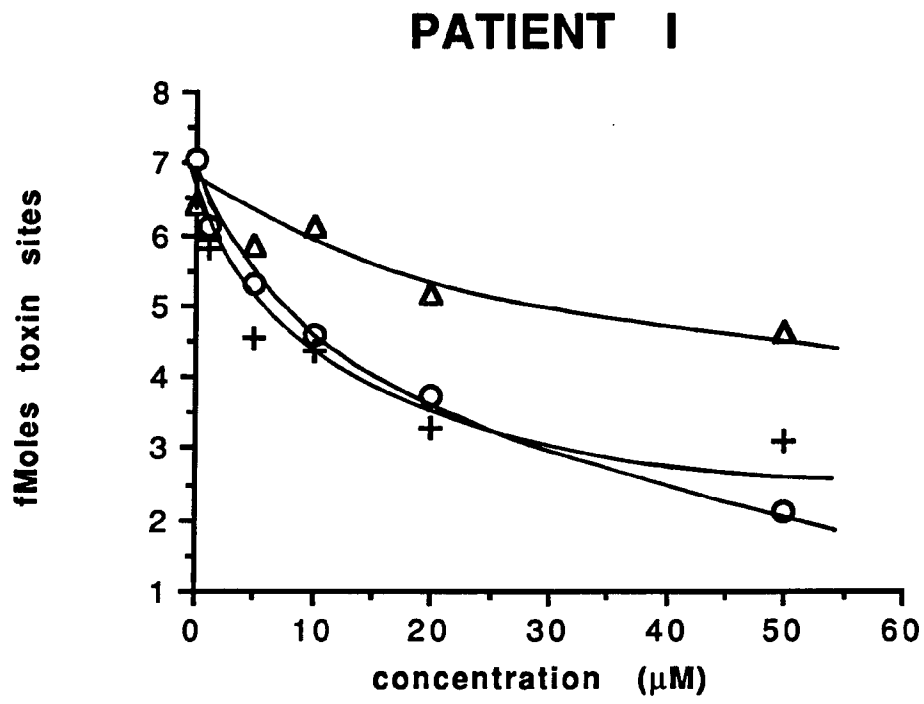
Flux recovery times were measured at 10 μM for both THA and physostigmine (Figure 6). Cells were preincubated for 30 min with either drug which was aspirated just before the flux stimulation step. The amount of drug remaining with the cells during the flux procedure was estimated to be as low as 10 nM and no more than 50 nM. The recovery phenomena demonstrated by this negative jump relaxation step differed between THA and physostigmine. The THA exhibited a sigmoidal relationship between time and flux recovery while physostigmine showed a simple, nearly linear recovery with time.

Patch Clamp Studies

Treatment of the Data. Treatment of the data followed the methods and rationale laid out in the seminal paper on the actions of anesthetics on single channel behavior by Neher and Steinbach (1978). Molecules such as an anesthetic block the open channel in a voltage-dependent manner that can be represented schematically as a simple linear sequential model of channel blocking. The action of anticholinesterases on nAChR was compared to those of the anesthetic QX-222 using this model.

The appearance of single open channels in the absence of drugs other than 100 nM ACh was of square wave pulses, the occurrence and durations of which were distributed in a fashion expected for a Poisson process. The average magnitude of these channels was 50 pS, the amplitudes varied directly as a function of the membrane holding potential and did not change appreciably in the presence of anesthetic or anticholinesterases. The character of the open current traces was altered by the presence of QX-222 and anticholinesterases. In the presence of these drugs, the open wave form was interrupted by brief closings (Figure 7) which presumably resulted from the presence of a drug in the open mouth of the channel that obstructed the passage of current until the drug diffused away. The duration of these blocking events could be altered by adjusting the

Figure 3. Anticholinesterases inhibit anti-AChR antibody binding to nAChR. Serum from two patients were used to evaluate the effectiveness of PHY, AMB (plus mark), THA (triangle), and HP 029 (open circle). PHY showed no inhibition and is not depicted.


PATIENT I
fMoles toxin sites
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
concentration (μM)

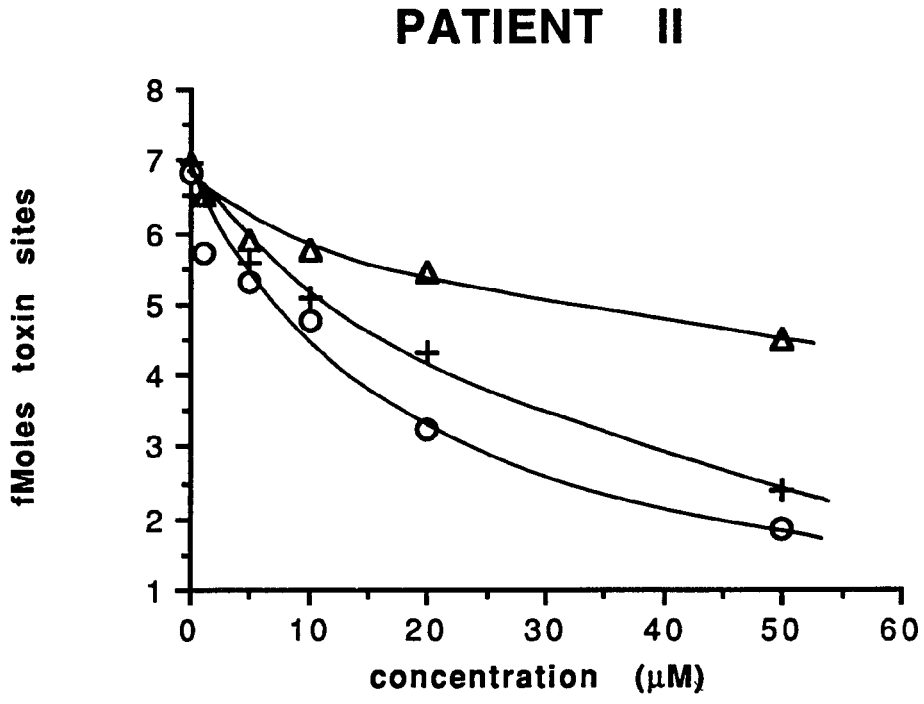

PATIENT II
fMoles toxin sites
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
concentration (μM)

Figure 4. THA inhibited carbamoylcholine-stimulated $^{22}Na^{+}$ influx into PC12 cells. Cells were either preincubated (open circle) with THA before flux stimulation or THA was coincubated (plus mark) with the agonist during the flux period. Fluxes were 30 sec at 24 °C. The data from each experiment were fitted to a single exponential from which an IC_{50} was calculated.

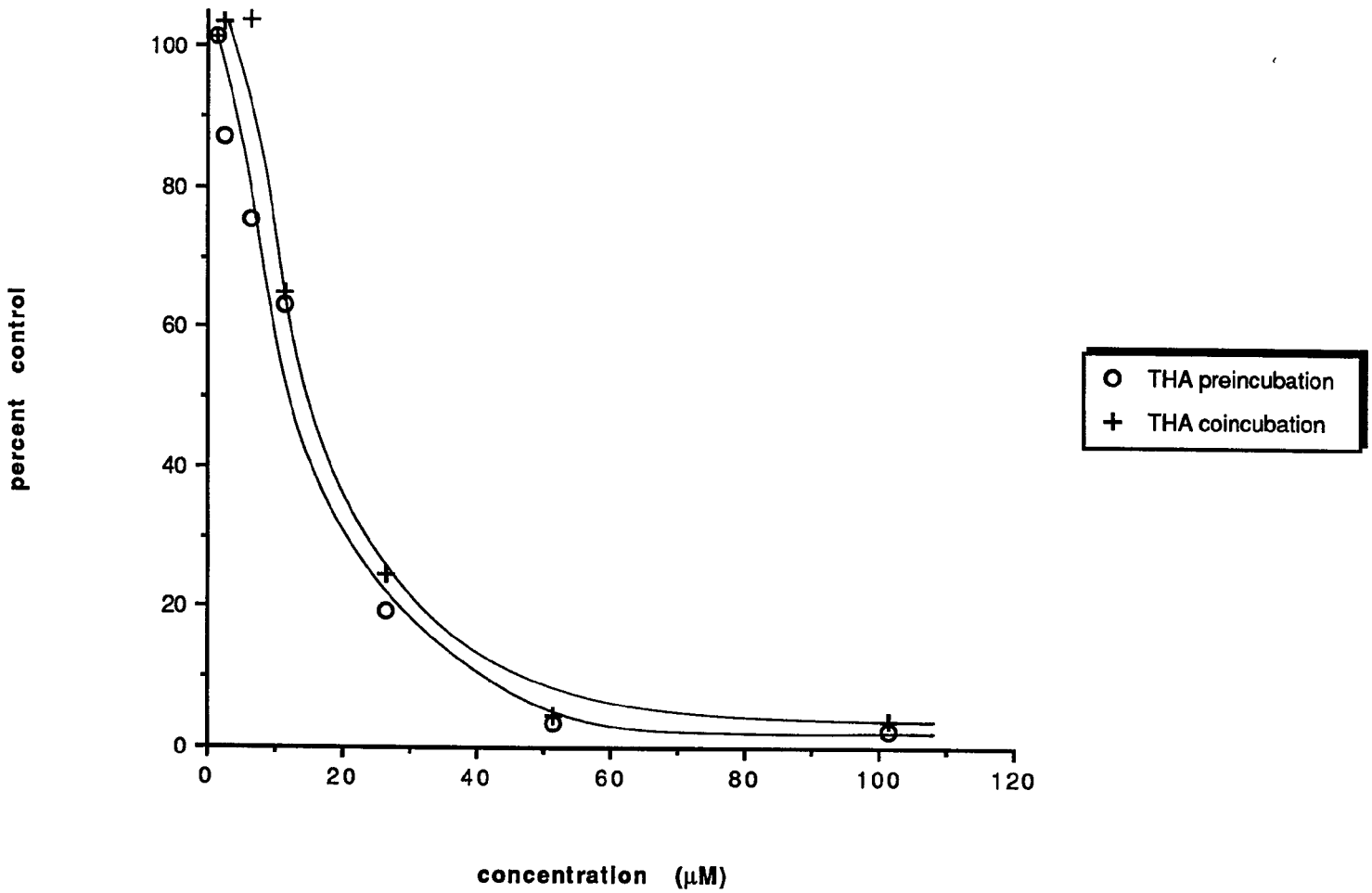

percent control
100
80
60
40
20
0
0
20
40
60
80
100
120
concentration (μM)
THA preincubation
THA coincubation

Figure 5. PHY inhibited carbamoylcholine-stimulated $^{22}Na^{+}$ influx into PC12 cells. Cells were either preincubated (open circle) with PHY before flux stimulation or PHY was coincubated (plus mark) with the agonist during the flux period. Fluxes were 30 sec at 24 °C. The data from each experiment was fitted to a single exponential from which an IC_{50} was calculated.

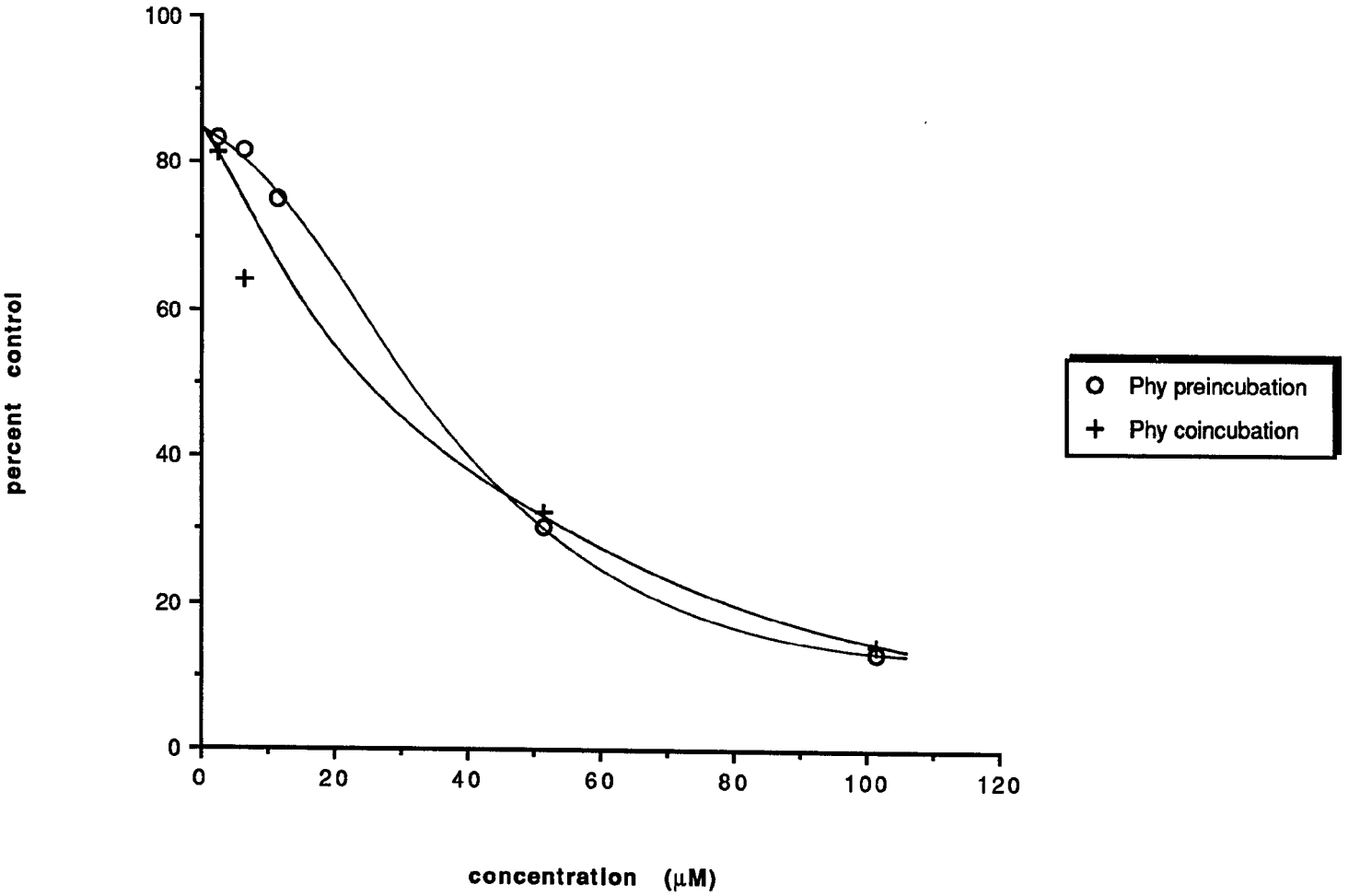
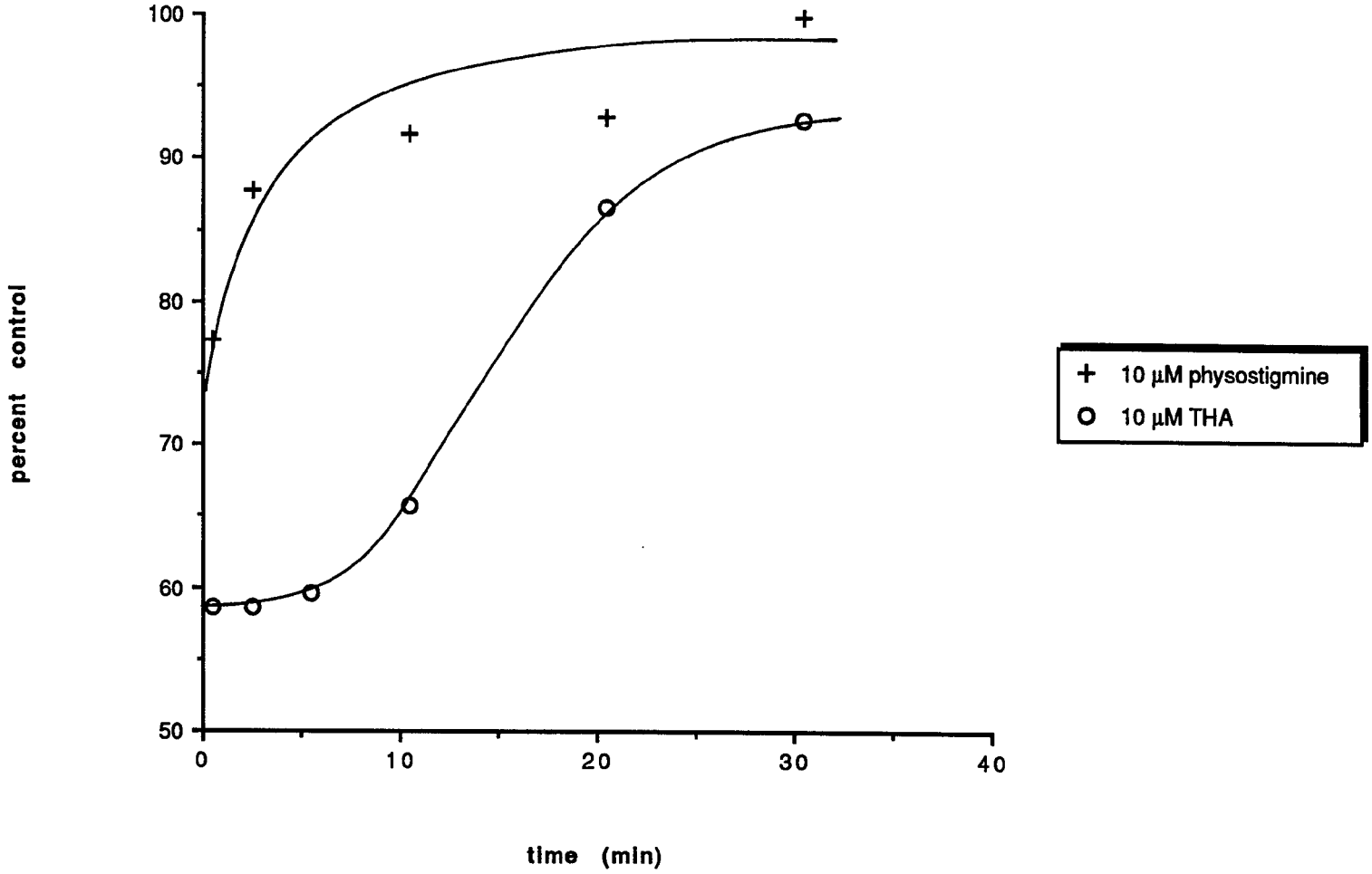

100
80
60
40
20
0
0
20
40
60
80
100
120
percent control
concentration (μM)
Phy preincubation
Phy coincubation

Figure 6. Carbamoylcholine-stimulated $^{22}Na^{+}$ influx recovered in PC12 cells after 30 min preincubation with either 10 μM PHY (plus mark) or 10 μM THA (open circle). Fluxes were 30 sec at 24 °C.


100
90
80
70
60
50
0
10
20
30
40
percent control
time (min)
+ 10 µM physostigmine
O 10 µM THA

membrane potential or, in the case of physostigmine and THA, drug concentration. These voltage-dependent and concentration-dependent phenomena were examined by simple plots of mean channel open times, closed times within a burst, burst open times, drug concentrations, and membrane potentials. These plots revealed the kinetic parameters of opening rate constants, forward, and backward rate constants for channel blocking in the presence of a drug.

Mean Channel Open Time. Mean channel open times exhibited voltage-dependence both in the presence and in the absence of anticholinesterases and QX-222. Control recordings were made in the presence of 100 nM ACh only. The mean channel open time in controls increased as membrane voltage increased (Figure 8). This relationship followed a simple exponential form revealed by plotting mean channel open times against pipette holding potentials. The channel closing rate was found to undergo an e-fold change per 113 mV change in holding potential. An important feature of channel blocking drugs is that they reverse this increase in channel open time with membrane hyperpolarization. In Figure 8, low concentrations of QX-222 were observed to decrease the mean channel open time in a concentration-dependent manner that was more pronounced with higher concentrations of drug. At 1 μM, the mean channel open time at 70 mV and 85 mV was not appreciably decreased but with hyperpolarization, there was no increase in mean channel open time such that it effectively reversed the trend seen in the absence of QX-222. With increasing concentrations of QX-222, mean channel open times decreased significantly with membrane hyperpolarization. QX-222 decreased the mean channel open time by 5.6-fold at 140 mV and 50 μM.

Physostigmine and THA were similar to QX-222 in their effects on mean channel open time. Physostigmine and THA prevented the hyperpolarization-induced increase in mean channel open time but their effects were quantitatively different from each other as well as from QX-222. THA was not as potent at high concentrations as QX-222 and physostigmine (Figure 9). At 140 mV and 50 μM THA, there was a 3.8-fold reduction in

mean channel open time. Hyperpolarization-induced increase in mean channel open time was prevented by 1 μM THA but not with 1 μM physostigmine (Figure 10). It was prevented by 10 μM physostigmine which reduced mean channel open time by 2.6-fold compared to 3.3-fold for 10 μM THA. Physostigmine proved to be the most potent drug at higher concentrations in reducing the mean channel open time (Figure 10). At 50 μM and 140 mV, there was an 8.5-fold reduction in mean channel open time. Beyond this concentration, physostigmine, THA, pyridostigmine, and ambenonium reduced channel activity to such a level as to make analysis impractical.

Mean Channel Burst Time. A closed time histogram was constructed for each data record at a particular holding potential. These histograms revealed at least two and usually three components comprising the durations of the closed times between events. These components were grouped as long, intermediate, and short closed times. Short closed times usually represented those times that occurred within a burst of openings of a single channel and presumably were the result of brief current blocking events, or gaps, due to the presence of a drug in the open mouth of a channel. A minimum time value between bursts, the critical time, was calculated as described previously using the intermediate and long time constants and was assigned to each record. Closed times shorter than this value dictated that the two events on either side of the closed time stretch should be counted as part of a burst of activity by a single channel opening. Closed times longer than this value indicated an interval between bursts or individual channel openings.

Both QX-222 and the anticholinesterases exhibited a voltage-dependent effect on the mean burst length. With membrane hyperpolarization, there was an increase in the mean burst length. THA and physostigmine exhibited a strong concentration effects; the higher the drug concentration, the longer the durations of the bursts. There was no significant concentration-dependence of burst length for QX-222. In Figure 11, QX-222 increased burst duration only 1.1- to 1.3-fold for 35 μM and 10 μM QX-222, respectively. For each concentration of drug, there was a voltage-dependent increase in burst duration.

Figure 7. Schematic representation of nAChR currents. The bottom solid line represents the baseline current while the top solid line represents the peak current at a particular holding potential. The horizontal hatched line represents half the average peak current and is the threshold through which an event (an opening or a closing trace) must traverse in order to be accepted as an event for analysis. There are two classifications of openings and closings; either long or short. Short closures are gaps and occur within a burst of openings by a single channel. Long closures demarcate the time between bursts or even some single openings. Bursts of activity consist of long or short openings separated by brief closing events.

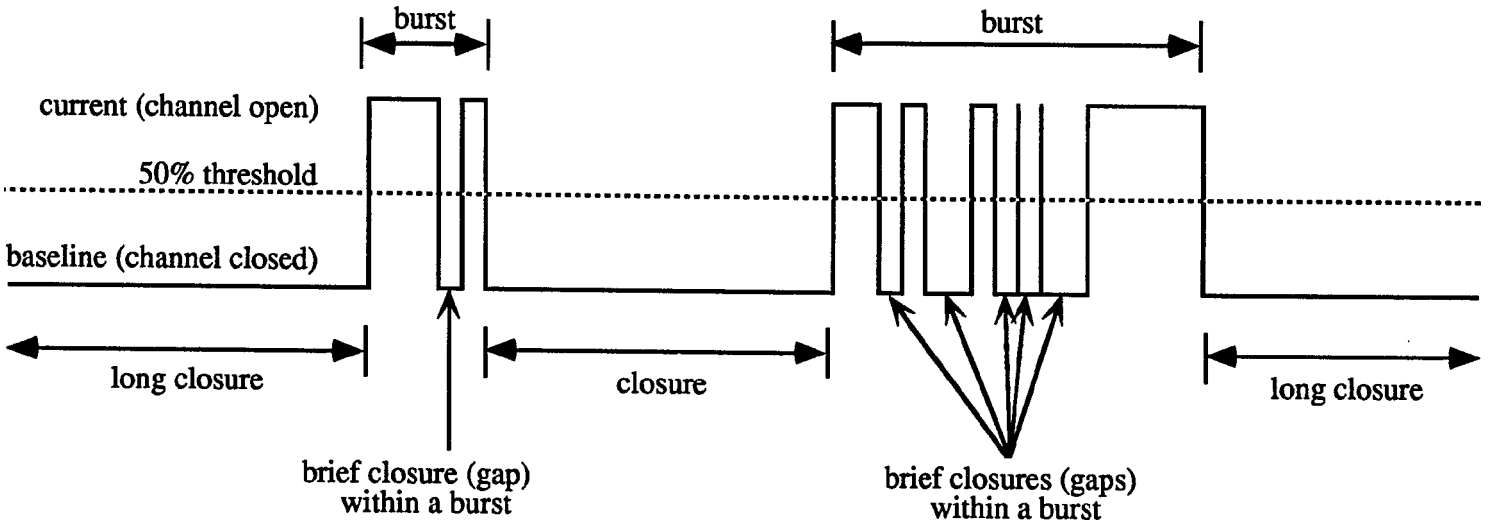

burst
burst
current (channel open)
50% threshold
baseline (channel closed)
long closure
closure
long closure
brief closure (gap)
within a burst
brief closures (gaps)
within a burst

Figure 8. Mean channel open time versus holding potential in the presence of QX-222. Holding potentials ranged from 70 mV to 140 mV. The control patch (hatch mark) contained only 100 nM ACh and the data is the sum of 3,645 events. QX-222 concentrations were 1 μM (square), 20 μM (triangle), 35 μM (plus mark), and 50 μM (open circle). The sum of events is 18,694.

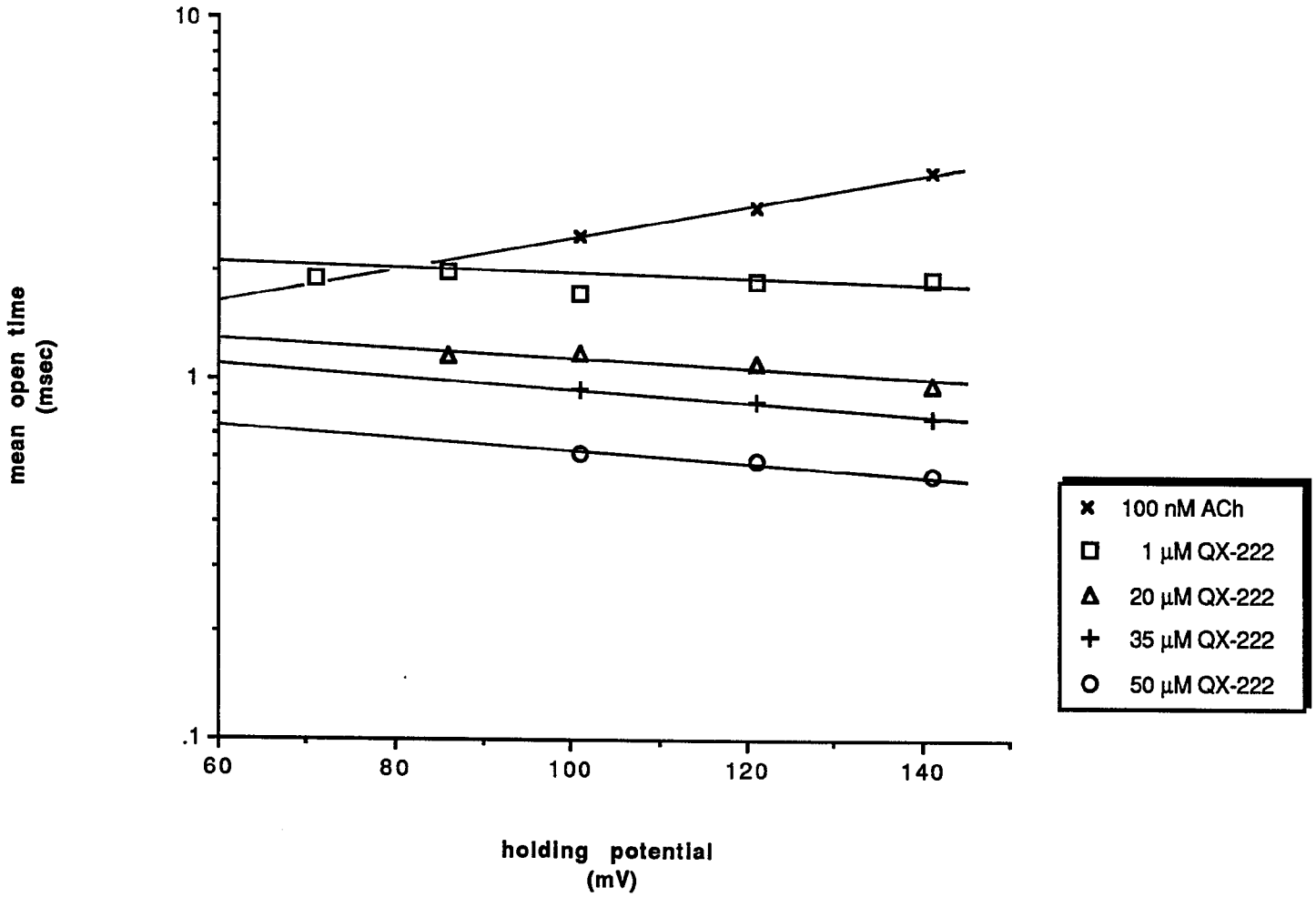

10
1
.1
mean open time
(msec)
60
80
100
120
140
holding potential
(mV)
100 nM ACh
1 μM QX-222
20 μM QX-222
35 μM QX-222
50 μM QX-222

Figure 9. Mean channel open time versus holding potential in the presence of THA. The total number of events represented is 34,127. The holding potentials ranged from 70 mV to 140 mV. The concentrations of THA were 1 μM (square, inset), 10 μM (plus mark), 20 μM (triangle), and 50 μM (open circle). All concentrations reversed the voltage-dependent increase in channel open time as seen in a control patch containing only 100 nM ACh. An experiment using 1 μM THA was performed with its own control (inset).

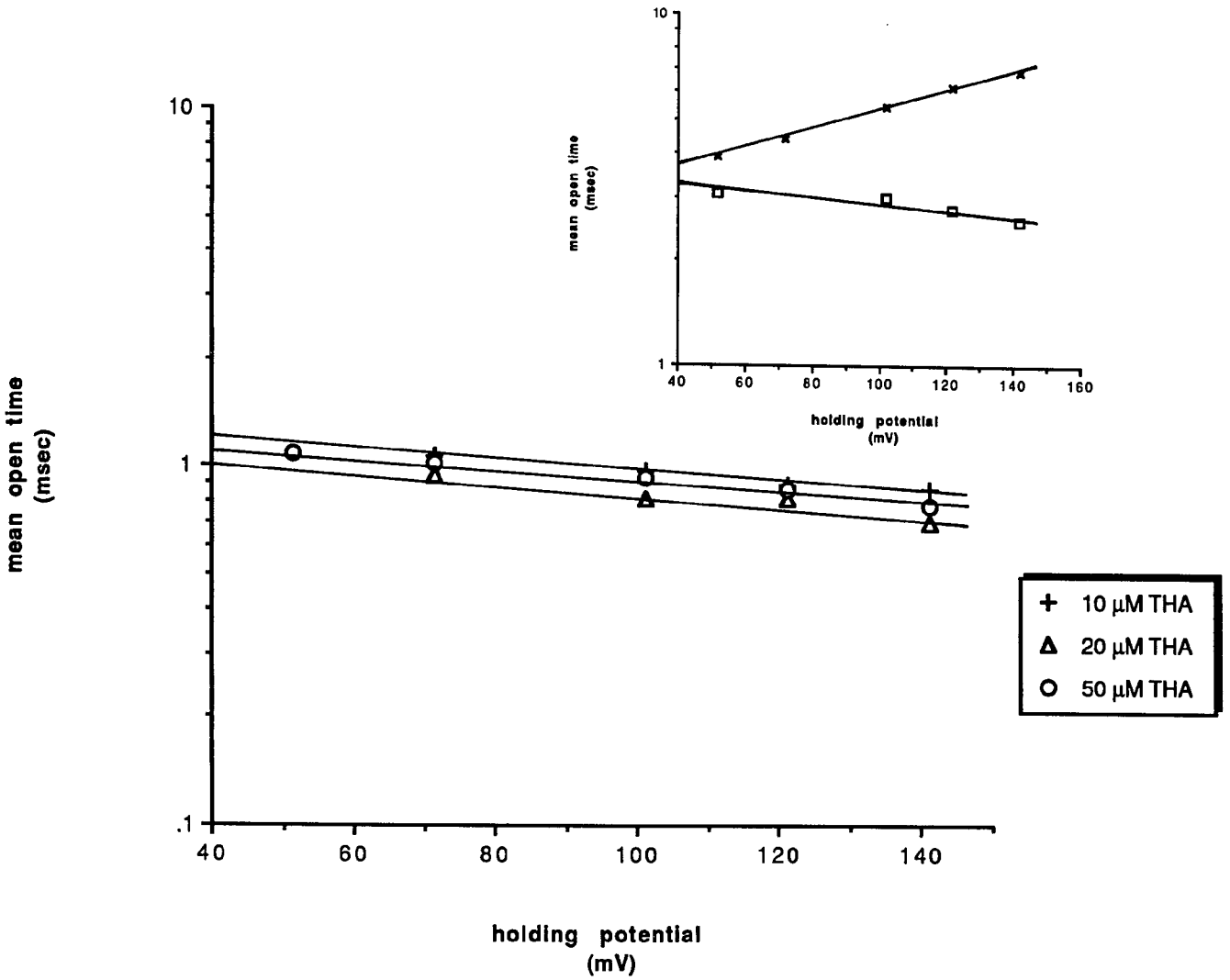
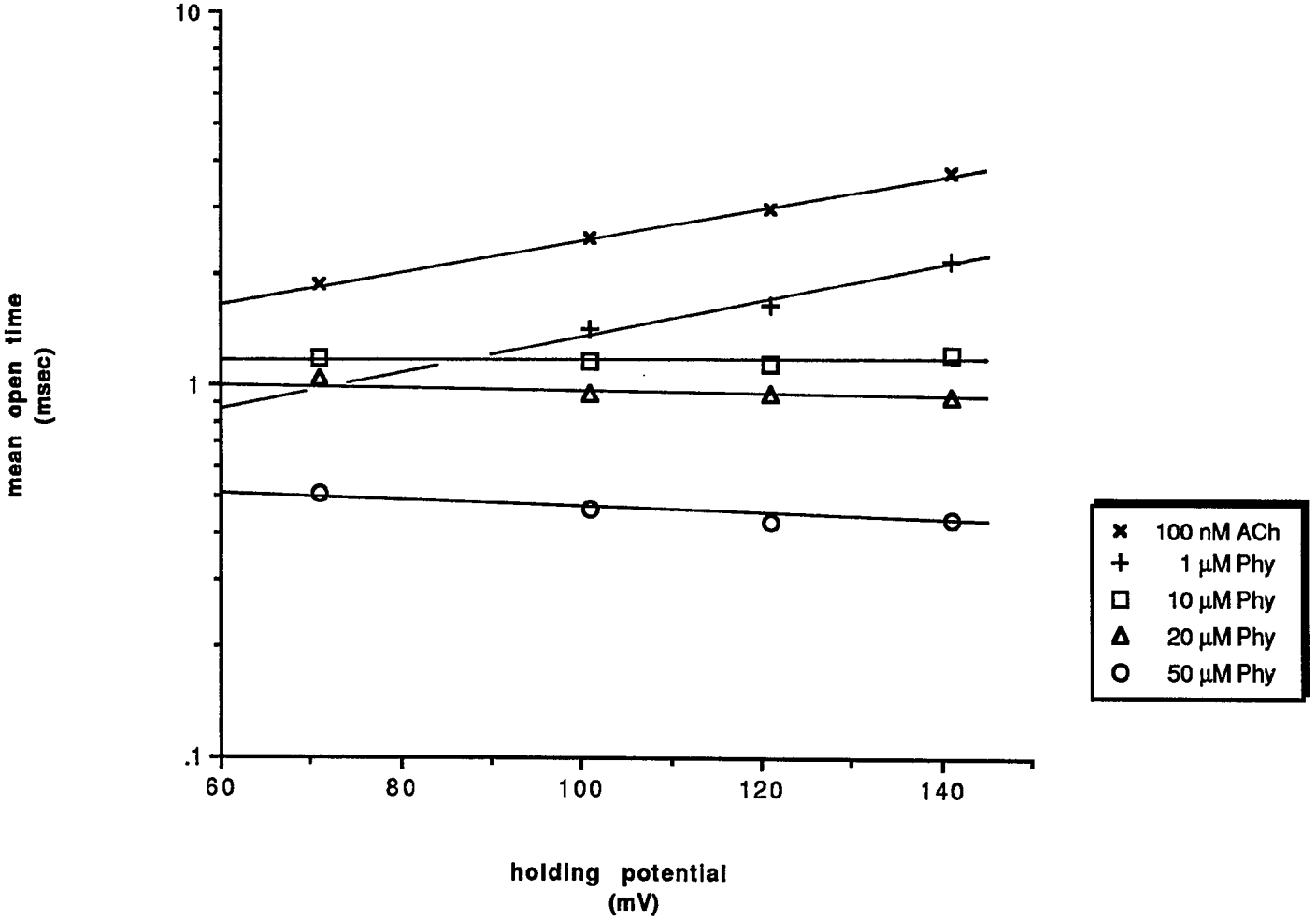

mean open time
(msec)
10
1
.1
40
60
80
100
120
140
holding potential
(mV)
10 μM THA
20 μM THA
50 μM THA
mean open time
(msec)
10
1
40
60
80
100
120
140
160
holding potential
(mV)

Figure 10. Mean channel open time versus holding potential in the presence of physostigmine. Holding potential values ranged from 70 mV to 140 mV. The concentrations of physostigmine were 1 μM (square), 10 μM (plus mark), 20 μM (triangle), and 50 μM (open circle). The total number of events represented is 22,958. Note that 1 μM physostigmine does not reverse the voltage-dependent increase in channel open time.


10
1
.1
60
80
100
120
140
mean open time
(msec)
holding potential
(mV)
100 nM ACh
1 µM Phy
10 µM Phy
20 µM Phy
50 µM Phy

THA was observed to increase mean burst duration up to 3.71-fold at 50 μM and 140 mV, the most extreme case (Figure 12). Physostigmine (Figure 13) increased the mean burst length 10.1-fold at 50 μM and 140 mV. Within each group of holding potentials, there was a strong concentration effect with a 9-fold increase in burst duration from 1 μM to 50 μM physostigmine at 140 mV. Physostigmine also exhibited a marked concentration-dependent increase in burst duration within each group of holding potentials. In contrast, this trend was only slightly appreciable in experiments using THA but was not apparent in experiments using QX-222.

Mean Channel Block Time. Within each burst, the brief closing events should represent the presence of a molecule in the open mouth of the channel that temporarily blocks the passage of current. The duration of these events should be independent of the concentration of drug present since they are able to diffuse away freely and stochastically. In Figure 14, the mean channel block time in the presence of QX-222 was plotted against the pipette holding potential. These blocked times displayed a strong voltage-dependence with an e-fold change in the blocking rate per 94 mV increase in membrane potential, but there was no clear trend with respect to the concentration of QX-222. The data were therefore grouped for linear regression analysis. The equation of the line was used to calculate the unblocking rate constant (Table 1).

The blocking phenomena by THA (Figure 15) and physostigmine (Figure 16) were strikingly different from QX-222. Not only did physostigmine and THA demonstrate the predicted voltage-dependent effect on the mean blocked time, but an unpredicted concentration-dependent effect as well. With increasing drug concentration, the duration of the blocking events was significantly prolonged. The voltage-dependence for block duration differed significantly between THA and physostigmine. An e-fold change in $k_f(V)$ occurred for every 280 mV for 1 μM THA but for only 62 mV for physostigmine. At 50 μM THA and physostigmine, an e-fold change in $k_f(V)$ occurred for every 84 mV and 45 mV, respectively. Thus, the voltage range to produce a given change in $k_f(V)$ was

very wide for THA but very narrow for physostigmine, with the most pronounced voltage sensitivity belonging to physostigmine.

Rate Constants Calculations. In order to calculate the rate constants for the forward rate of channel blocking, the open time reciprocal (sec^{-1}) at each voltage step was plotted against the concentration of drug used. The y-intercept on these plots is the channel closing rate constant, $\alpha(V)$, and the slope of the lines fitted to the data was used to give the forward rate of channel blocking, $k_f(V)$, directly in units of $M^{-1}sec^{-1}$. For illustrative purposes, the effect of QX-222 on mean channel open time is presented in Figure 17. It was demonstrated that, as predicted, the mean channel open time decreased with increasing concentration of drug and increasing membrane holding potential. Calculations utilizing the slopes of the individual lines showed that the forward rate of channel blocking increased as a function of voltage (Table 1) but that no real difference was demonstrated for closing rate constant (Table 4). Similar effects were demonstrated by THA as illustrated in Figure 18. The forward rate of channel blocking in the presence of THA increased with increasing membrane holding potential (Table 2) while the rate of channel closing became much slower. The channel behavior in the presence of physostigmine (Figure 19) was also similar to QX-222 and THA in that the forward rate of channel blocking increased with increasing membrane holding potential (Table 3) while the rate of channel closing became slower (Table 4).

The forward blocking rate constants were dissimilar for all drugs tested. In order from fastest to slowest $k_f(V)$ was THA, physostigmine, and QX-222. The forward blocking rate at 70 mV for THA (Table 2) may be high but is listed for computational purposes. THA demonstrated a significantly faster rate of blocking than physostigmine or QX-222. At 100 mV, $k_f(V)$ for THA was 4.9-fold that for QX-222 and was 1.9-fold that for physostigmine.

As listed in Table 4, the closing rates did not follow a smooth trend with respect to holding potential for QX-222 and physostigmine. The closing rate constants for THA

Figure 11. Mean channel burst time versus holding potential in the presence of QX-222. Concentrations of QX-222 were 1 μM (dark cross-hatch), 10 μM (dark stippled), 20 μM (light cross-hatch), and 35 μM (white bar). The control data is composed of 3,240 events and the experimental data is composed of 5,566 events of a single channel. The burst times represent open times that occur in groups separated from one another by a minimum calculated closed time period. The burst times within a group defined by the holding potential were shorter than a control (black bar) in which there was no QX-222 present. The burst times were voltage-dependent and increased with increasing holding potential.

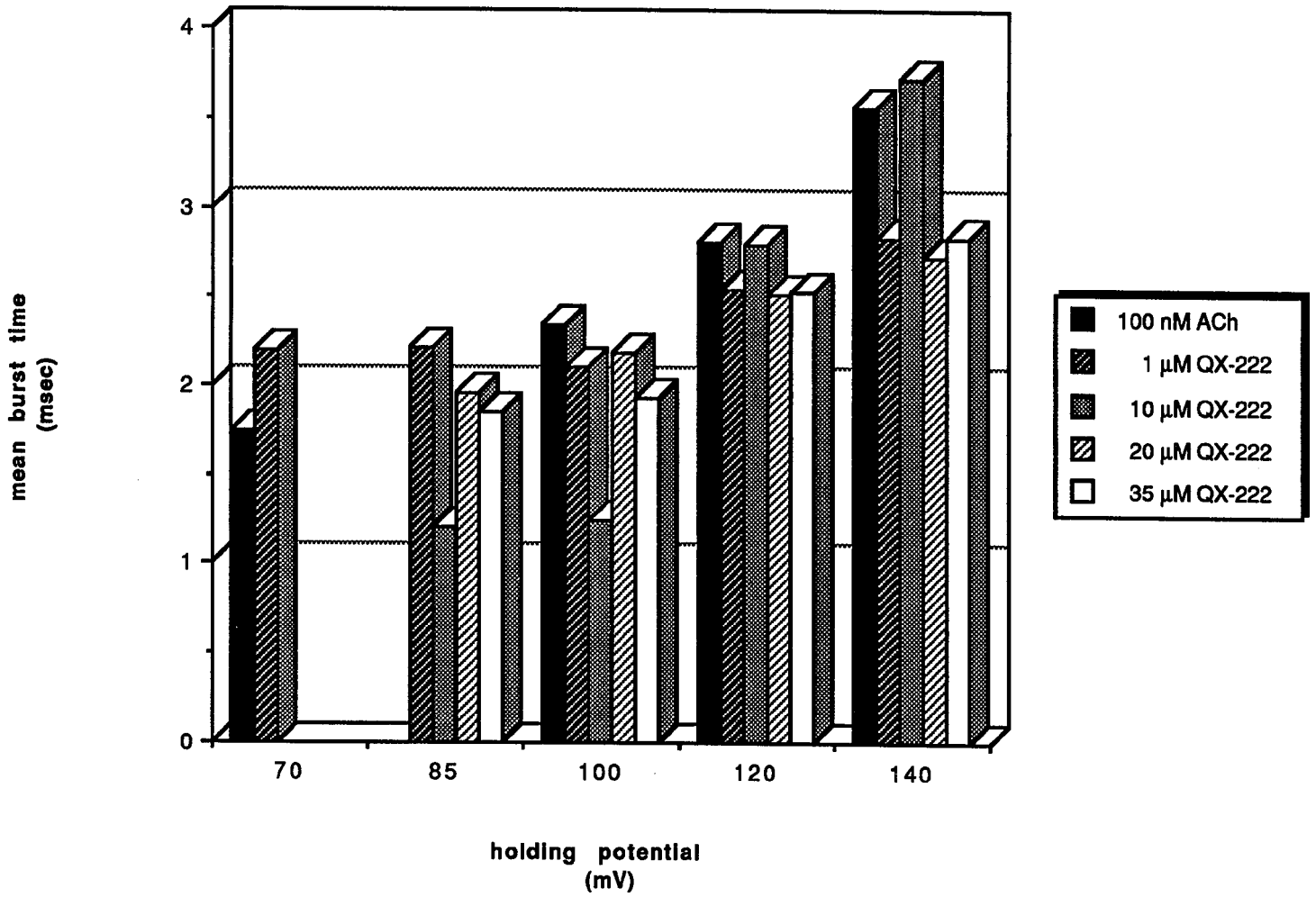

mean burst time (msec)
4
3
2
1
0
70
85
100
120
140
holding potential (mV)
100 nM ACh
1 µM QX-222
10 µM QX-222
20 µM QX-222
35 µM QX-222

Figure 12. Mean channel burst time versus holding potential in the presence of THA. The control (black bar) is that depicted in Figure 11. The concentrations of THA depicted are 1 μM (dark cross-hatch), 10 μM (dark stippled), 20 μM (light cross-hatch), and 50 μM (white bar). The data for THA is composed of 14,307 events. Mean burst time is significantly increased compared to both control values and QX-222. Mean burst time increased with respect to increases in drug concenration but showed little trend with respect to holding potential.

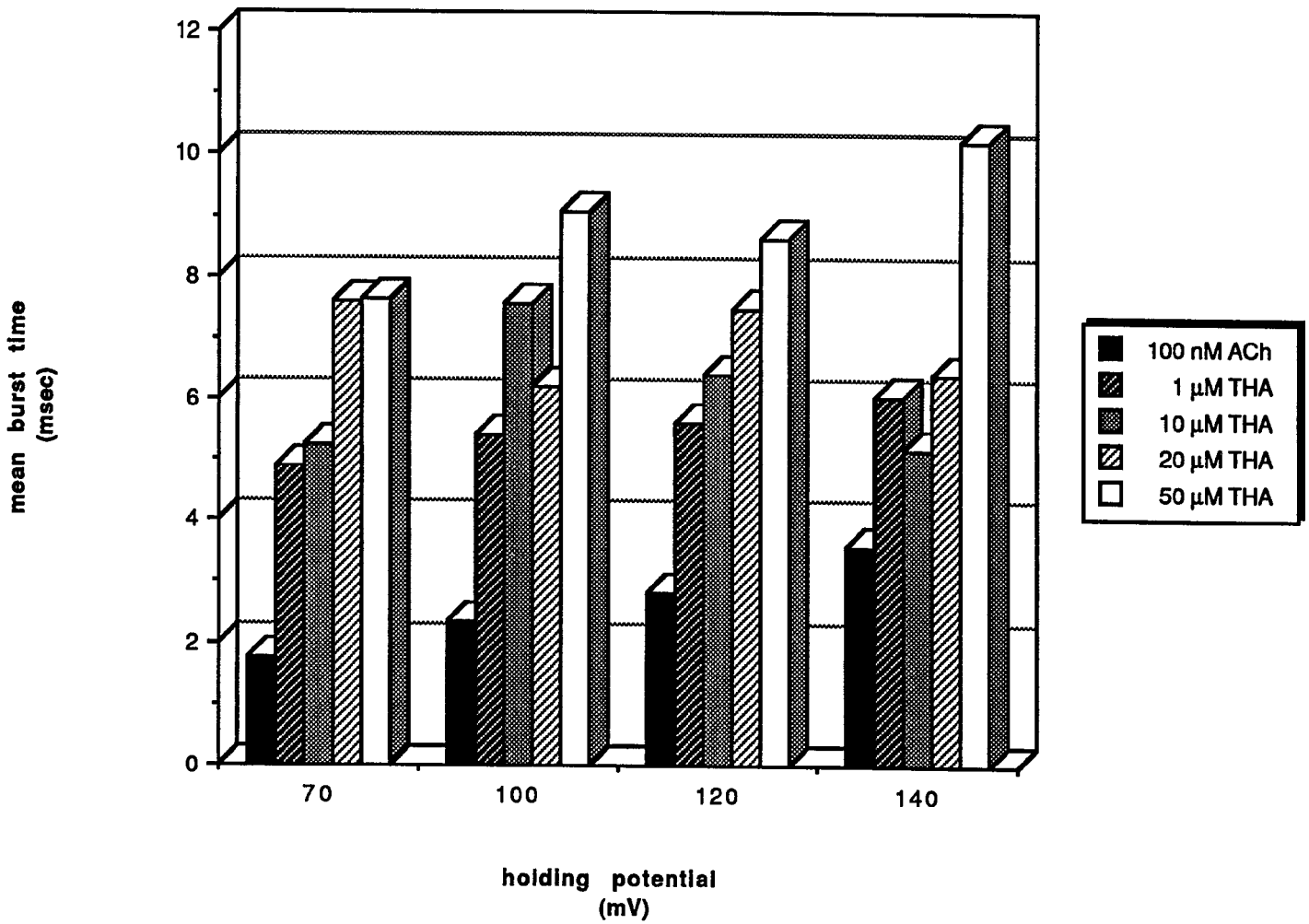

12
10
8
6
4
2
0
mean burst time
(msec)
70
100
120
140
holding potential
(mV)
100 nM ACh
1 μM THA
10 μM THA
20 μM THA
50 μM THA

Figure 13. Mean channel burst time versus holding potential in the presence of physostigmine. Concentrations of physostigmine were 1 μM (dark cross-hatch), 10 μM (stippled), 20 μM (light cross-hatch), and 50 μM (white bar). Control values (black bar) are those used in Figure 14. The data is comprised of 10,937 total events. The mean burst durations are greatly increased compared to both QX-222 and THA. Within groups defined by holding potential there is a clear increasing trend in burst duration with respect to drug concentration.

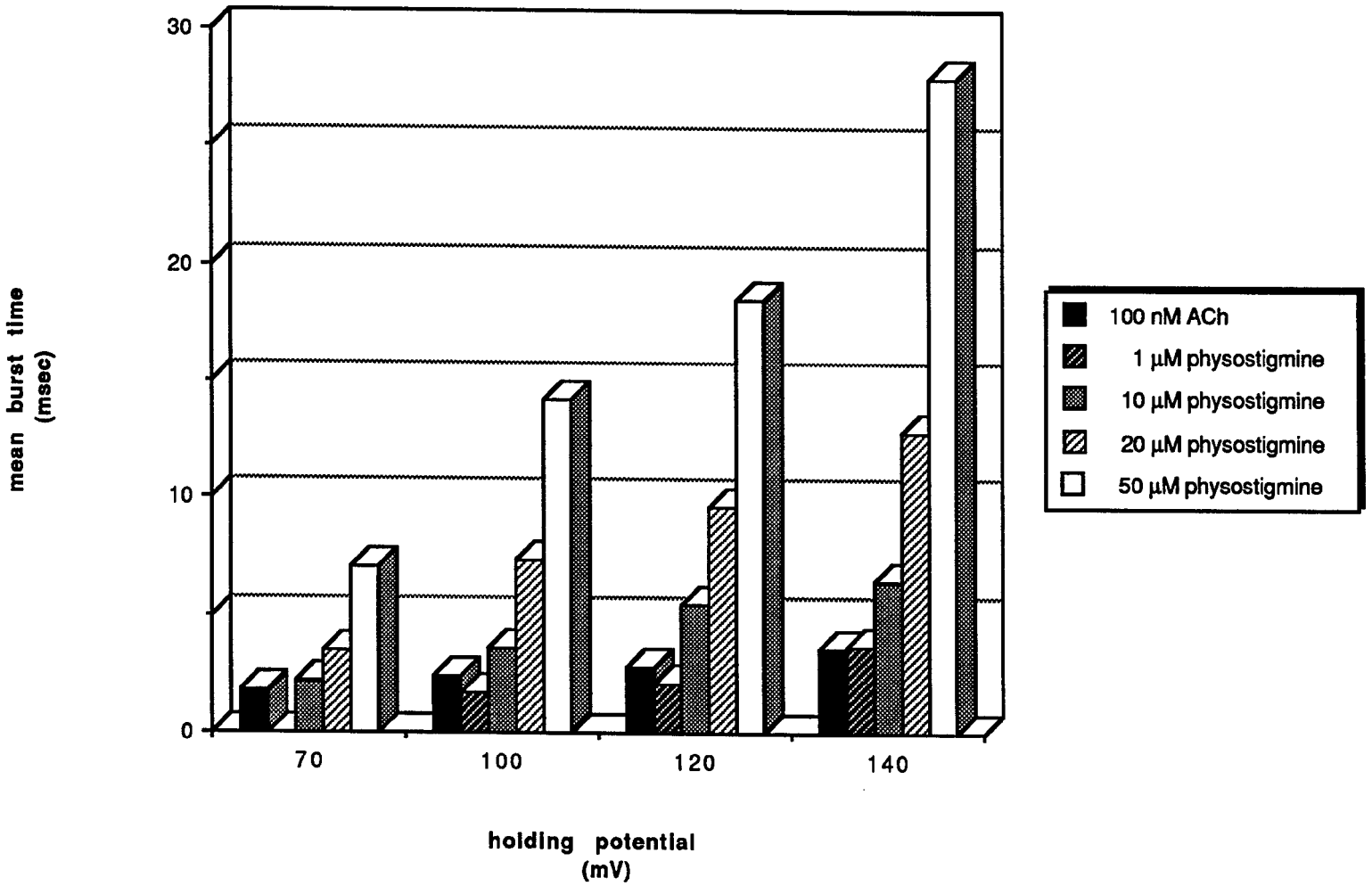

30
20
10
0
mean burst time (msec)
70
100
120
140
holding potential (mV)
100 nM ACh
1 µM physostigmine
10 µM physostigmine
20 µM physostigmine
50 µM physostigmine

Figure 14. Mean channel block time versus holding potential in the presence of QX-222. Concentrations of QX-222 were 1 μM (square), 10 μM (plus mark), 20 μM (triangle), 35 μM (open circle), and 50 μM (hatch mark). These data represent the short closed times during a burst of channel openings. There was no clear trend for channel block time with respect to drug concentration. After the data were grouped, a regression line fit was constructed to show the voltage-dependent increase in channel block time due to the presence of QX-222. The slope of the regression line is the value of the backward rate constant of the blocking reaction.

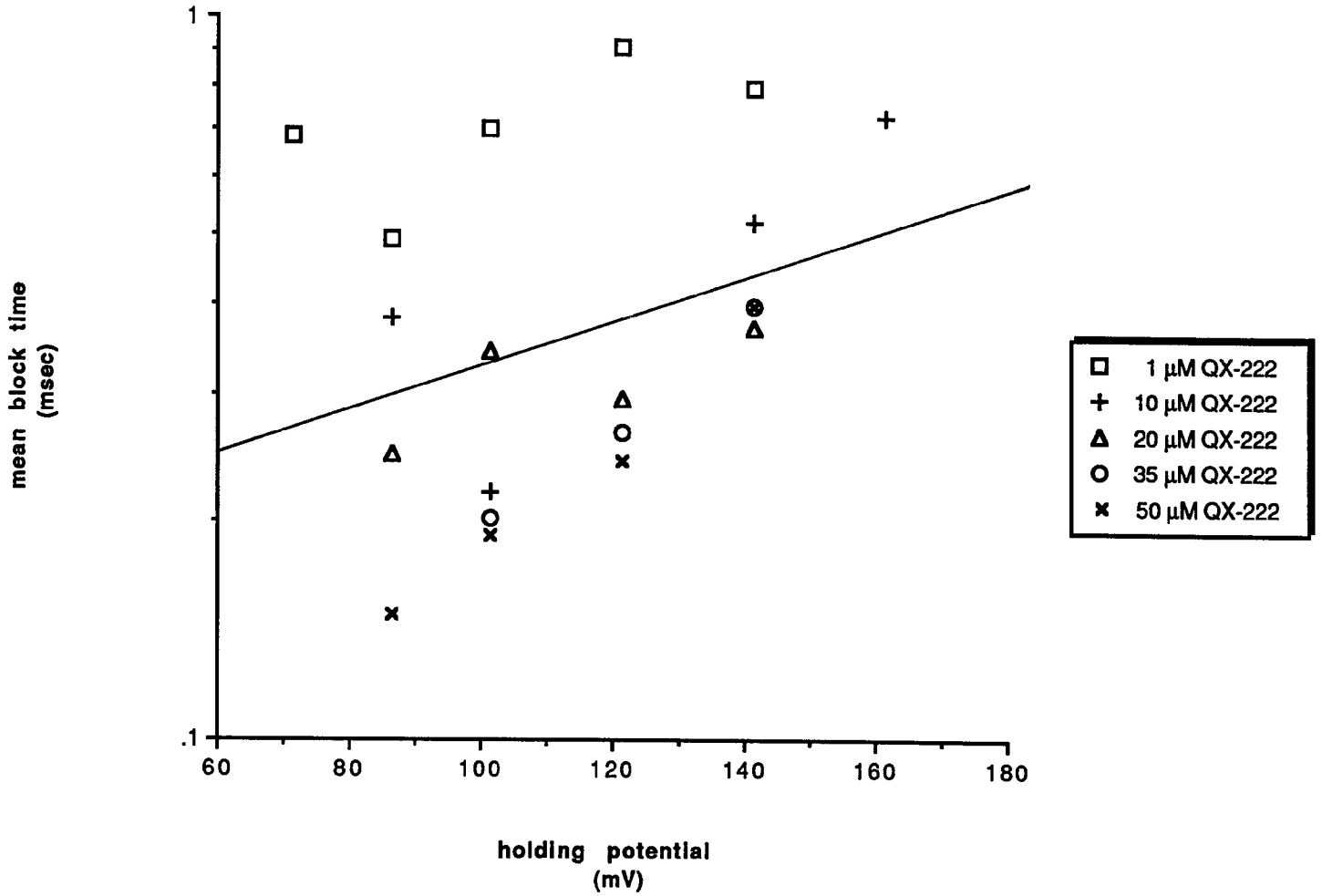

1
.1
60
80
100
120
140
160
180
mean block time
(msec)
holding potential
(mV)
1 µM QX-222
10 µM QX-222
20 µM QX-222
35 µM QX-222
50 µM QX-222

Table 1

Forward and Backward Rate Constants of Blocking by QX-222

k_f ($10^6 M^{-1} sec^{-1}$)	k_b (sec^{-1})	k_b/k_f (μM)	potential (mV)
24.5	2275	93	140
23.3	2615	112	120
15.2	3005	198	100

Figure 15. Mean channel block time versus holding potential in the presence of THA. Concentrations of THA were 1 μM (square), 10 μM (plus mark), 20 μM (triangle), and 50 μM (open circle). There was a clear trend for channel block time with respect to concentration. A linear regression program was used to construct a line of best fit for each concentration. The graph illustrates the voltage-dependent and concentration-dependent increase in channel block time due to the presence of THA. The slope of this line represents the backward rate constant of the blocking reaction.

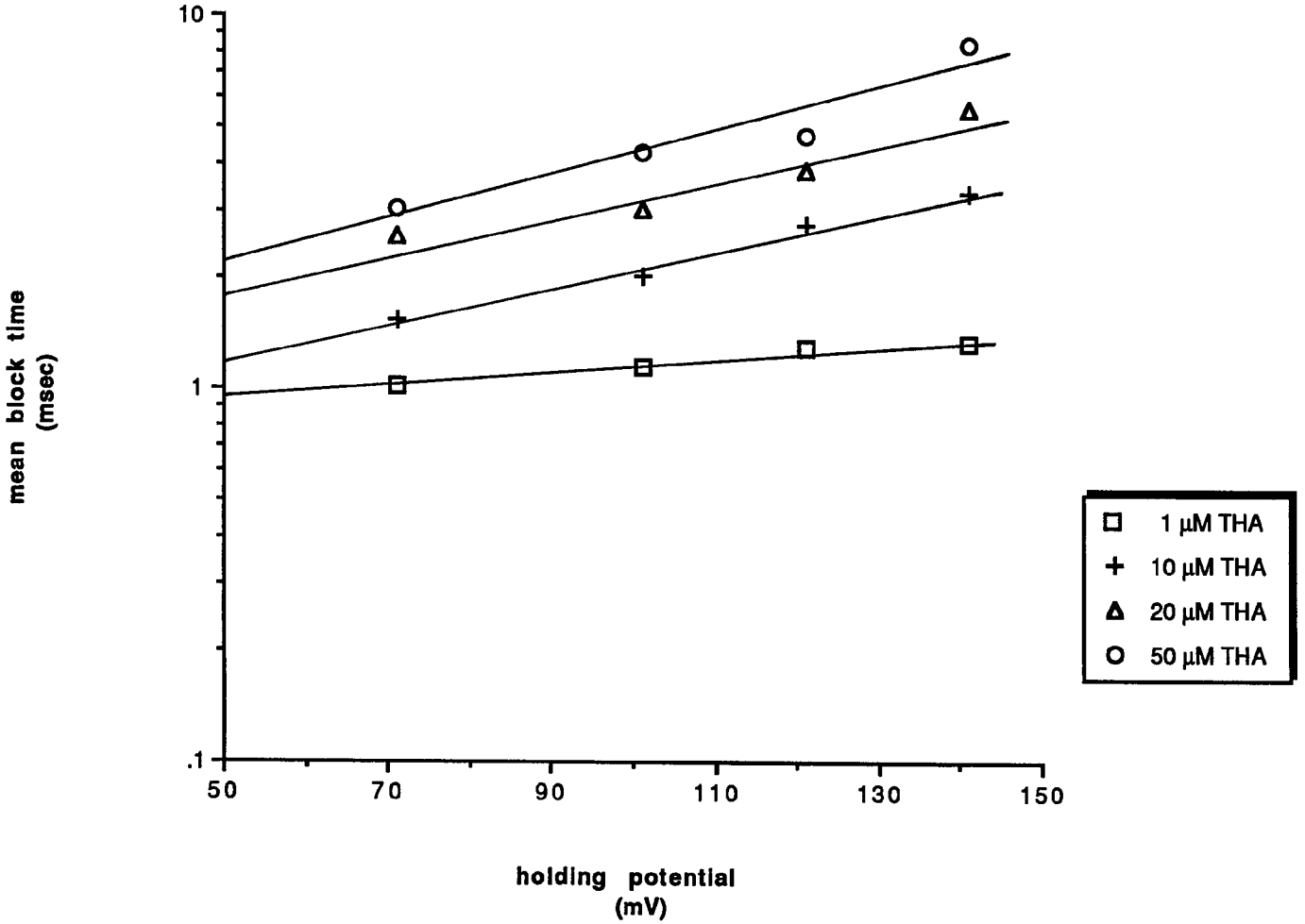
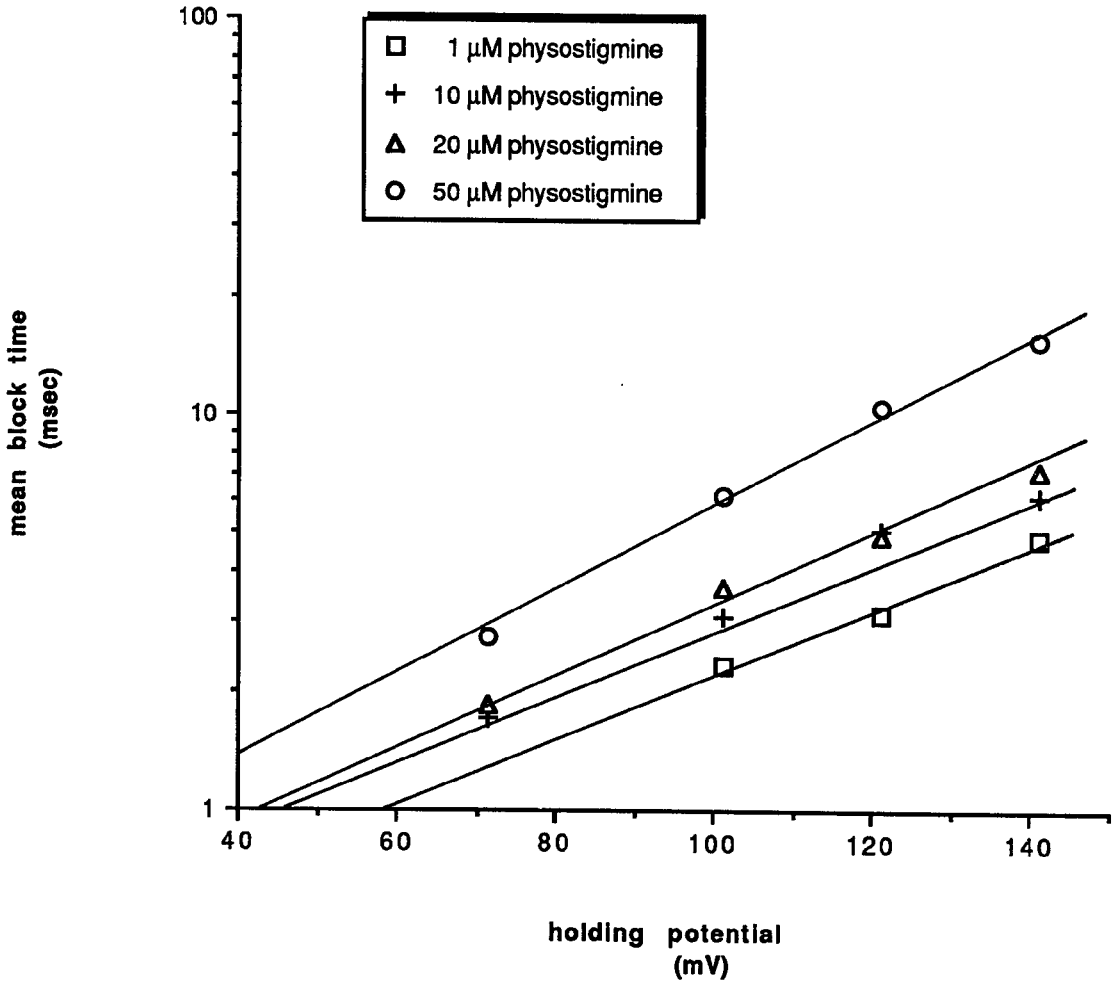

10
1
.1
mean block time
(msec)
50
70
90
110
130
150
holding potential
(mV)
1 µM THA
10 µM THA
20 µM THA
50 µM THA

Figure 16. Mean channel block time versus holding potential in the presence of physostigmine. Concentrations of physostigmine were 1 μM (square), 10 μM (plus mark), 20 μM (triangle), and 50 μM (open circle). Similar to THA, physostigmine produced channel block that not only increased as a direct function of membrane voltage but increased with respect to concentration as well. The duration of channel block was increased overall compared to QX-222 and THA.


100
10
1
40
60
80
100
120
140
1 μM physostigmine
10 μM physostigmine
20 μM physostigmine
50 μM physostigmine
mean block time (msec)
holding potential (mV)

showed a clear relationship to membrane potential in that with hyperpolarization, $\alpha(V)$ became much slower. The range of the channel closing rates was narrow for QX-222 and physostigmine and the cell-to-cell patch variance may have obscured their differences.

The individual backward rates of channel blocking, $k_b(V)$, were calculated by plotting the mean channel block time versus pipette holding potential. The plots were fitted exponentially and the resulting equations were used to calculate the rates directly using the relationship predicted from the sequential blocking model by Equation 2. As predicted, QX-222 exhibited concentration-dependence for k_f, and k_b was concentration-independent (Table 1). The unblocking rate constants for THA (Table 2) and physostigmine (Table 3) were both dependent upon the concentration of drug present. In order from fastest to slowest $k_b(V)$ was QX-222, THA, and physostigmine. Comparisons at 50 μM and 100 mV revealed that $k_b(V)$ for QX-222 was 12-fold greater than that for THA and 29-fold greater than that for physostigmine. The unblocking rate for 50 μM THA at 100 mV was 2.3-fold greater than that for physostigmine under the same conditions.

The equilibrium dissociation constant, K_d, for the interaction between drug and receptor can be calculated simply by dividing the unblocking rate constant by the blocking rate constant. The K_d for all drugs tested were voltage-dependent and increased with decreasing membrane potential. In order from highest to lowest K_d was QX-222, THA, and physostigmine. The K_d for QX-222 ranged over 2-fold (Table 1) while THA and physostigmine exhibited much larger ranges. The K_d ranged over 6.9-fold for THA (Table 2) and 15-fold for physostigmine (Table 3). At 100 mV and 1 μM drug concentration, the K_d for THA and physostigmine were similar at 14.8 μM and 9.5 μM, respectively. However, physostigmine exhibited a stronger voltage-dependence than THA since, for example, at 140 mV and 1 μM drug concentration, the K_d ranged down to 4.5 μM for physostigmine while the K_d for THA was 11.9 μM.

Figure 17. Plot of the concentrations of QX-222 versus the open time reciprocal. Each category of data represents one holding potential and the mean channel open times that were recorded at that potential for the various concentrations of drug that were used. Within each category the mean channel open time reciprocal (sec^{-1}) was plotted against the drug concentration. From such a plot, the forward rate constant of channel blocking can be derived. Two holding potentials are displayed, 120 mV (open circle) and 140 mV (plus mark).

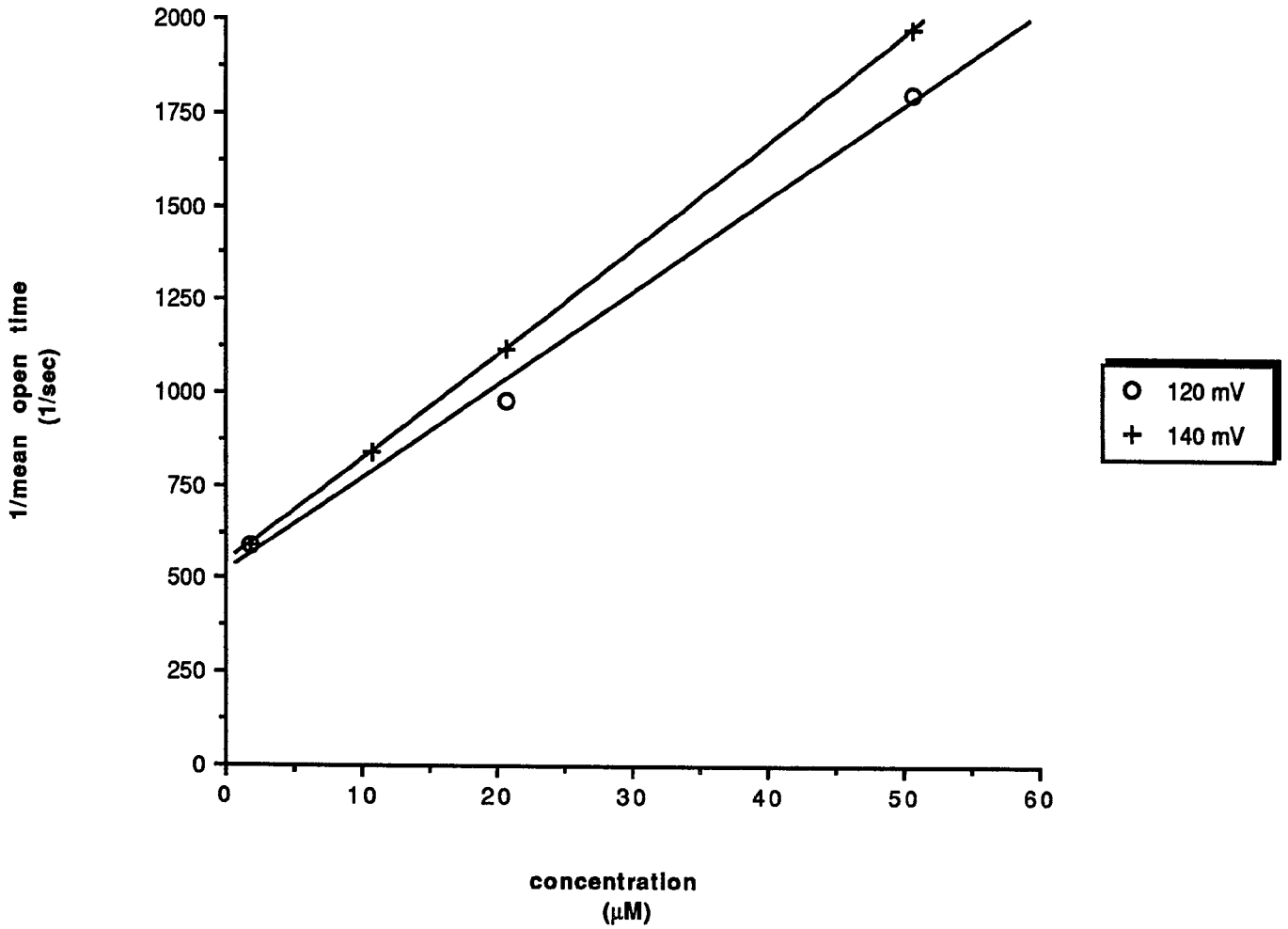

2000
1750
1500
1250
1000
750
500
250
0
0
10
20
30
40
50
60
1/mean open time
(1/sec)
concentration
(μM)
120 mV
140 mV

Table 2

Forward and Backward Rate Constants of Blocking by THA

concentration (μM)	k_f (10^6 $M^{-1}sec^{-1}$)	k_b (sec^{-1})	k_b/k_f (μM)	potential (mV)
50	65.8	142	2.16	140
	63.6	187	2.94	120
	62.1	245	3.95	100
	73.3	368	5.02	70
20	65.8	204	3.10	140
	63.6	254	4.00	120
	62.1	318	5.12	100
	73.3	444	6.06	70
10	65.8	312	4.72	140
	63.6	393	6.18	120
	62.1	497	8.00	100
	73.3	704	9.60	70
1	65.8	782	11.9	140
	63.6	849	13.3	120
	62.1	920	14.8	100
	73.3	1039	14.2	70

Table 3

Forward and Backward Rate Constants of Blocking by Physostigmine

concentration (μM)	k_f ($10^6 M^{-1} sec^{-1}$)	k_b (sec^{-1})	k_b/k_f (μM)	potential (mV)
50	39.0	40	1.026	140
	36.5	64	1.753	120
	32.7	103	3.150	100
	30.8	209	6.786	70
20	39.0	99	2.54	140
	36.5	142	3.89	120
	32.7	205	6.27	100
	30.8	354	11.49	70
10	39.0	108	2.77	140
	36.5	156	4.27	120
	32.7	224	6.85	100
	30.8	389	12.63	70
1	39.0	175	4.49	140
	36.5	232	6.36	120
	32.7	309	9.45	100
	30.8	474	15.39	70

Table 4

Closing Rate Constants for QX-222, THA, and Physostigmine

QX-222	THA	physostigmine	potential (mV)
579	261	437	140
459	347	523	120
577	392	613	100
	415	512	70

Rates are in sec^{-1} units

Figure 18. Plot of the concentrations of THA versus the open time reciprocal. Holding potentials are 100 mV (square), 120 mV (open circle), 140 mV (plus mark). The ordinate units are in sec^{-1}. Construction and analysis is the same as for Figure 17.

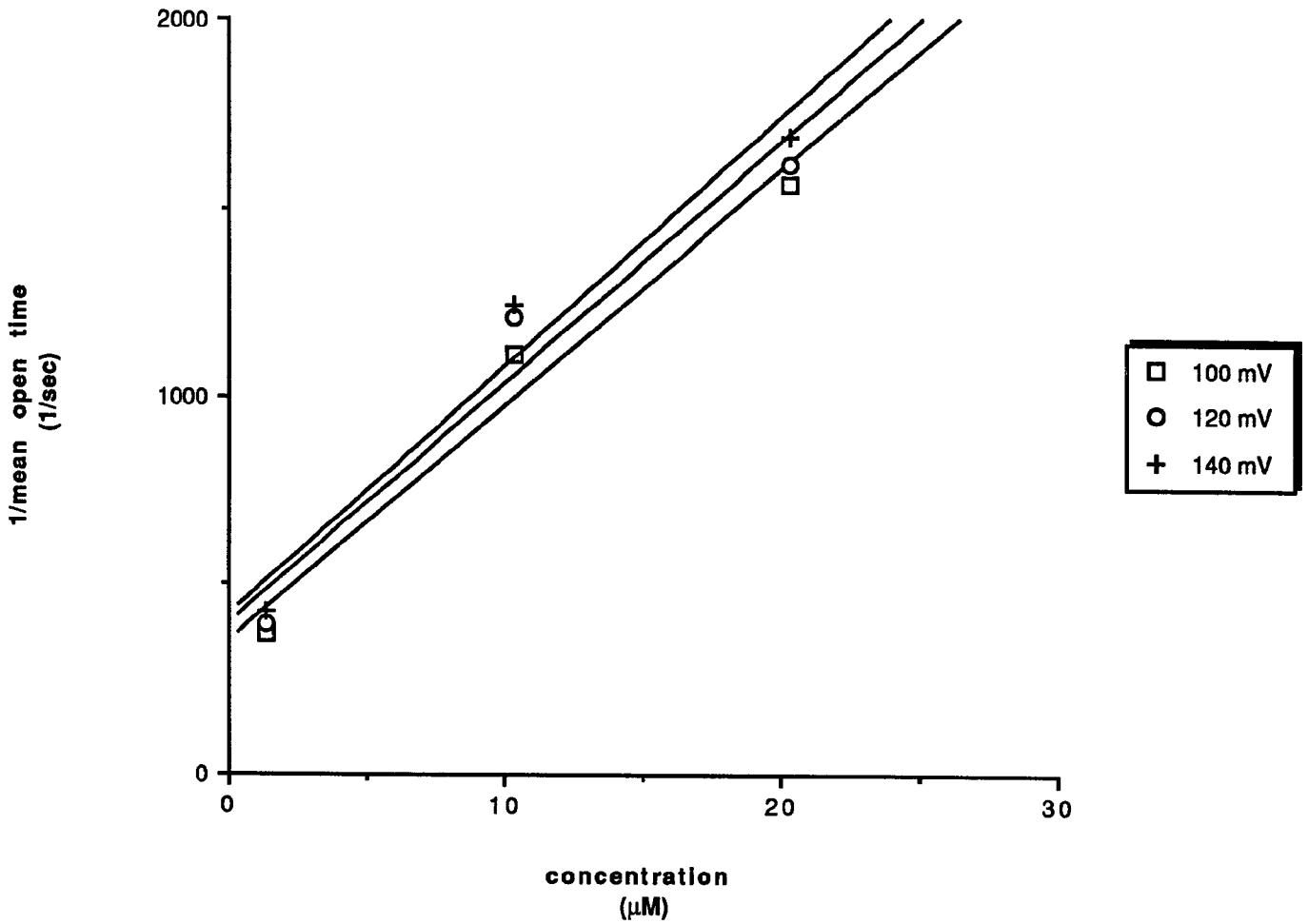
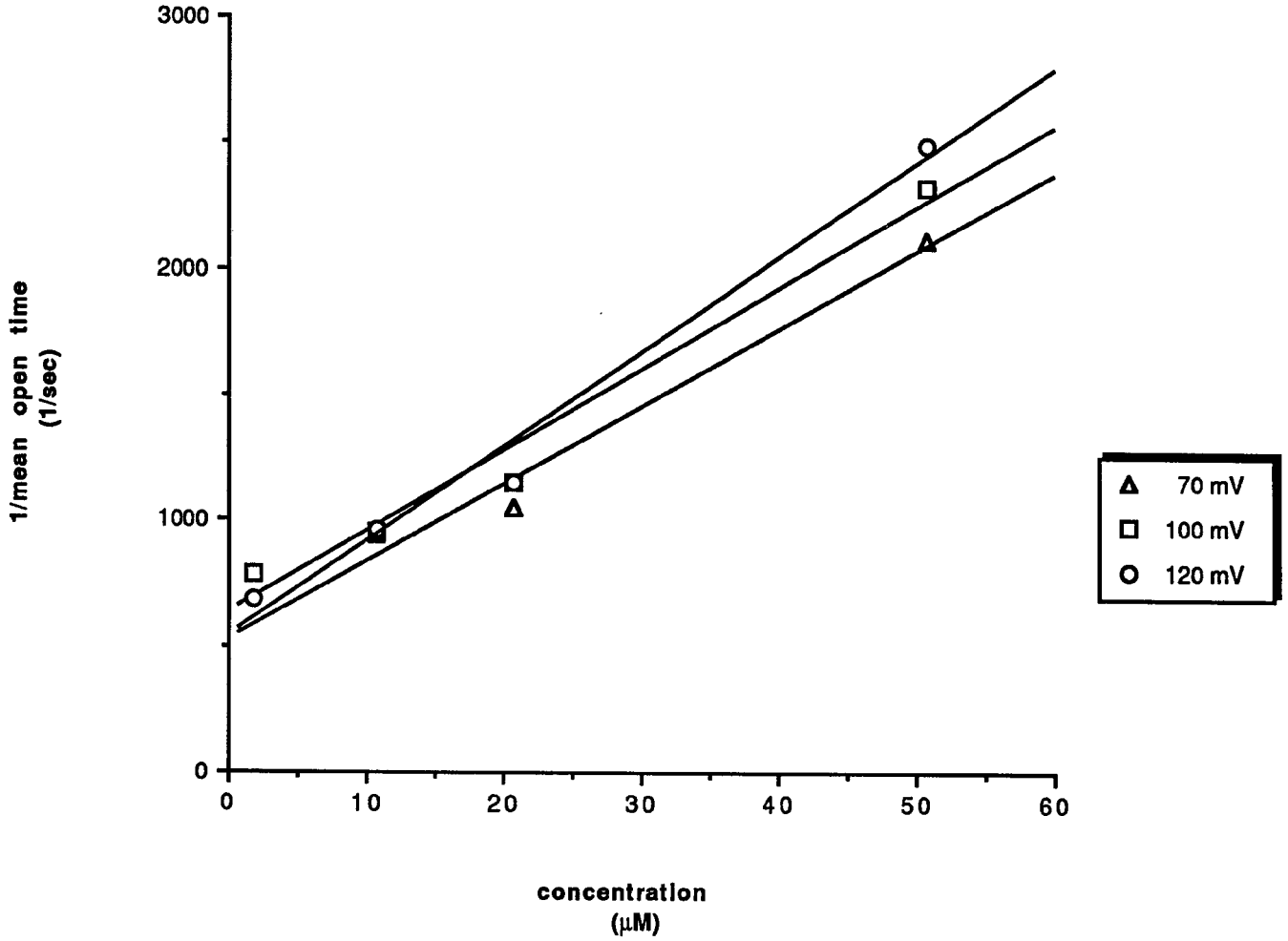

2000
1000
0
0
10
20
30
1/mean open time
(1/sec)
concentration
(μM)
100 mV
120 mV
140 mV

Figure 19. Plot of the concentrations of physostigmine versus the open time reciprocal. The ordinate units are in sec^{-1}. Construction and analysis is the same as for Figure 17. Holding potentials are 70 mV (triangle), 100 mV (square), 120 mV (open circle).


3000
2000
1000
0
1/mean open time (1/sec)
0
10
20
30
40
50
60
concentration (μM)
70 mV
100 mV
120 mV

With the introduction of a blocking drug into the environment of active receptor channels, the relative proportions of long- and short-lived open or closed states would be predicted to change. Histograms of the frequency of occurrences of open or closed states of particular durations were constructed by computer and the outline of the histogram was fitted by regression analysis into one or more exponential curves, the peaks of which represented the opening or closing rates of the events beneath them. A summary of the opening rates for control channels only in the presence of 100 nM ACh is presented in Table 5. It shows that two populations of channel open times predominated, a fast population and a slower population. At 70 mV, the rates were not significantly different in that the slow channels opened at a rate of 2.03×10^{-3} sec^{-1} while the faster population opened at a rate of 2.46×10^{-3} sec^{-1}. However, the proportions of the two channel rate populations were significantly different in that 77% were of the slower variety. As the cell membrane was hyperpolarized, the proportion of faster channel openings rose slightly to 34% while at the same time the opening rate of the faster population increased sharply to 1.18×10^{-4} sec^{-1}, a 21-fold increase in the opening rate. The opening rate of the slower population decreased steadily but by only 2.2-fold.

The addition of QX-222, physostigmine, and THA in the patch pipette introduced new populations of opening and closing rates, usually splitting the fast component into an intermediate and an even faster component. The histogram opening rates summary for QX-222 in Table 6 demonstrates that alterations in rate components was concentration and voltage-dependent. At 85 mV and 1 μM QX-222 there are two opening rates with the slow component predominating at 81%. As the concentration of QX-222 was increased, an intermediate rate of about 1.0×10^{-3} sec^{-1} appeared. The most significant change in rates occurred as both concentration and membrane voltage increased. At 140 mV and 1 μM QX-222, three opening rates were clearly distinguished that coalesced into a single fast component when the concentration of QX-222 reached 35 μM. This is consistent with the idea that as the membrane is hyperpolarized, the charged anesthetic experiences a greater

force pushing it into the mouth of the channel and thus blocking it at a statistically higher frequency and for a longer time. Since the channel cannot close until the blocking drug has diffused away, the channel is kept in the open state for a longer time and passes current intermittently as the blocking molecule dances in the mouth of the open channel, creating a flickering pattern of brief open and closed states, a burst. The distribution of burst lengths is likewise dependent upon both the membrane potential and the concentration of blocking drug. Table 7 presents a summary of burst analysis opening rates of nAChR channels in the presence of QX-222. At 1 μM QX-222 and at 85 mV, the opening rates were not significantly different from the opening rates and population proportions presented for the same conditions in Table 6, indicating that under these conditions QX-222 did not significantly alter channel behavior. However, as the concentration was increased, bursts of openings occurred containing basically two opening rates of roughly equal proportions. This indicated that, as predicted, the longer channel openings were broken up into shorter, more frequent openings. As the cell membrane was hyperpolarized this basic phenomenon did not change and, as in the controls, the slower rates became even slower by about 1.5-fold.

The presence of QX-222 produced long bursts of openings with very brief closings within them. The closed time histograms of these records revealed groups of very fast and slower rates. A summary of the closing rates for QX-222 burst analyses appears in Table 8 and clearly demonstrates the overwhelming preponderance of short-lived closings that were the result of brief channel blocking events by QX-222. At 35 μM QX-222 and at 140 mV, the rate of channel blocking was very fast, and no slow blocking events were recorded.

Similar analyses for THA and physostigmine were undertaken. These drugs produced more complex patterns of blocking activity than QX-222. Table 9 presents a summary of the the opening rates for THA. The rates and proportions of populations were very similar to those demonstrated by QX-222 in Table 6. However, THA produced a

larger population of the fast openings at a much lower concentration than did QX-222 as illustrated by comparing the two drugs, for example, at 85 mV. At 85 mV and 1 μM THA, 42% of the openings were very fast at 2.33 x 10^{-4} sec^{-1} compared to only 19% at 2.32 x 10^{-4} sec^{-1} for QX-222 under the same conditions. The opening rates compiled for burst analysis for channels in the presence of THA (Table 10) were very different from those for QX-222. At 1 μM QX-222 and at 85 mV, two populations of opening rates were described in Table 7, 80% being of the slow variety. In the presence of THA under similar conditions, that population falls to just 46%. As both concentration and membrane potential were increased, that figure drops dramatically to just 21% at 140 mV and 50 μM THA. The opening rate under these conditions was also significantly reduced by up to 5-fold when comparing QX-222 and THA at 20 μM and 140 mV. This pattern was repeated under all conditions. The presence of THA produced significant populations of intermediate components of the opening rates within bursts. These components never comprised more than one-third the total population of opening rates.

The most striking difference between the effects of QX-222 and those of THA were changes in the channel closing rates. A summary of closing rates within bursts produced by THA is presented in Table 11. Very fast closing events comprised a majority of channel closings in the presence of QX-222 (Table 8) while in the presence of THA only an intermediate rate was seen. At 1 μM THA, a very fast closing component occurred to a significant degree at 70 mV. As the cell membrane was hyperpolarized, that component shifted into an intermediate component that comprised from 56% to 68% of the closing rates demonstrated. At even higher concentrations of THA, the very fast component disappeared, the intermediate component became less than 15% of the population of closing rates, and the slow component dominated by up to 92%. This demonstrated a voltage-dependent and a concentration-dependent channel block by THA.

The same complex phenomena displayed by channels in the presence of THA also occurred in the presence of physostigmine, which was similar to THA in that intermediate

opening and closing rates appeared in its presence. Table 12 is a summary of opening rates for channels in the presence of physostigmine. At 1 μM physostigmine, the relative proportions of opening rates populations were not very different from those recorded for QX-222 in Table 6. However, as concentration was increased, a marked divergence occurred in that an intermediate component displaced both the very fast and the slow opening rate components, illustrated best at 20 μM physostigmine. At 100 mV and above, only the intermediate opening rate was present having displaced the slow component which comprised the major opening rate observed for QX-222 under similar conditions. Physostigmine and THA produced similar bursting patterns. The summary of opening rates within bursts in the presence of physostigmine is presented in Table 13, and the rates are very similar to those observed for THA in Table 10. At concentrations of THA above 20 μM the fast opening rate component begins to disappear whereas in the presence of physostigmine it persisted as a significant population ranging from about 20% to 45% of the components present.

The closing rates within bursts in the presence of physostigmine differed markedly at low concentrations from those observed in the presence of THA. Table 14 summarizes this data for physostigmine. At 1 μM physostigmine and all membrane potentials tested, virtually all the closing events within bursts were extraordinarily fast and comprised greater than 96% of the components present. The closing rates within bursts produced by THA as summarized in Table 11 were relatively slow and comprised less than 70% of the components present. As the concentration of physostigmine was increased, an intermediate component similar to that observed in the presence of THA and QX-222 appeared but accounted for less than 10% of the components at any time. As with THA, however, the largest closing rate population was the slow component. At concentrations of 10 μM and above for both physostigmine and THA, the slow component comprised 75% to 91% of the total component population. These differed significantly from those proportions observed with QX-222 where the fast component comprised greater than 90%

of the total component population as summarized in Table 8. In comparison with data from experiments using QX-222, these phenomena demonstrate that THA and physostigmine decreased the closing rates within bursts at the expense of the very fast components in a concentration-dependent manner. The fast component predominated under all conditions using QX-222.

Table 5

Histogram Opening Rates Summary for Acetylcholine

concentration (nM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
100	2.457×10^{-3} 2.031×10^{-3}	23 77	1.110×10^{-4} 2.876×10^{-3}	33 67	1.159×10^{-4} 3.482×10^{-3}	32 68	1.177×10^{-4} 4.529×10^{-3}	34 66

Table 6

Histogram Opening Rates Summary for QX-222

concentration (μM)	85 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1	2.324×10^{-4} 2.106×10^{-3}	19 81	1.037×10^{-4} 1.911×10^{-3}	29 71	7.200×10^{-5} 2.058×10^{-3}	33 67	2.834×10^{-5} 1.338×10^{-4} 2.125×10^{-3}	33 15 52
10	9.294×10^{-4}	100	8.268×10^{-5} 9.958×10^{-4}	21 79			9.263×10^{-5} 1.313×10^{-3}	33 67
20	1.028×10^{-3}	100	1.246×10^{-4} 1.291×10^{-3}	27 73	1.364×10^{-4} 1.236×10^{-3}	28 82	1.105×10^{-4} 9.903×10^{-4}	22 78
35	1.025×10^{-3}	100	8.269×10^{-4}	100	1.607×10^{-4} 8.532×10^{-4}	18 82	6.798×10^{-4}	100

Table 7

Histogram Opening Rates Summary for QX-222 Burst Analysis

concentration (μM)	85 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1	2.703×10^{-4} 2.545×10^{-3}	20 80	1.171×10^{-4} 2.695×10^{-3}	36 64	8.773×10^{-5} 3.289×10^{-3}	41 59	8.874×10^{-5} 3.863×10^{-3}	44 56
10	1.144×10^{-3}	100	9.479×10^{-5} 1.442×10^{-3}	29 71			1.127×10^{-4} 3.469×10^{-3}	33 67
20	3.299×10^{-4} 2.743×10^{-3}	40 60	1.503×10^{-4} 3.197×10^{-3}	45 55	1.839×10^{-4} 4.170×10^{-3}	50 50	1.100×10^{-4} 4.556×10^{-3}	57 43
35	4.712×10^{-4} 2.930×10^{-3}	49 51	6.543×10^{-4} 2.710×10^{-3}	43 57	1.834×10^{-4} 2.855×10^{-3} 4.816×10^{-2}	43 56 1	1.656×10^{-4} 4.259×10^{-3}	44 56

TABLE 8

Histogram Closing Rates Summary for QX-222 Burst Analysis

concentration (μM)	85 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1	4.268×10^{-5} 8.816×10^{-4}	80 20	1.464×10^{-5} 8.878×10^{-4}	96 4	2.926×10^{-5} 2.818×10^{-4} 1.690×10^{-3}	61 20 19	1.929×10^{-5} 8.180×10^{-4}	82 18
10	3.736×10^{-5} 1.088×10^{-3}	94 6	8.349×10^{-5} 6.913×10^{-4}	93 7			2.333×10^{-4} 2.901×10^{-3}	94 7
20	7.921×10^{-5} 3.326×10^{-3}	98 2	1.200×10^{-4} 5.642×10^{-3}	98 2	1.842×10^{-4} 2.718×10^{-3}	99 1	3.013×10^{-4}	100
35	9.616×10^{-5} 7.640×10^{-3}	89 11	9.093×10^{-5} 1.242×10^{-3}	98 2	1.742×10^{-4} 1.383×10^{-3}	98 2	3.060×10^{-4}	100

Table 9

Histogram Opening Rates Summary for THA

concentration (μM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1	2.333×10^{-4} 4.374×10^{-3}	42 58	1.544×10^{-4} 9.940×10^{-4} 4.449×10^{-3}	33 15 52	1.672×10^{-4} 3.617×10^{-3}	37 63	2.144×10^{-4} 3.320×10^{-3}	34 66
10	1.132×10^{-4} 1.117×10^{-3}	25 75	1.736×10^{-4} 1.267×10^{-3}	23 77	1.190×10^{-4} 1.005×10^{-3}	20 80		
20	5.183×10^{-4}	100	1.149×10^{-4} 6.826×10^{-4}	23 77	5.649×10^{-4}	100	2.347×10^{-4} 7.840×10^{-4}	49 51
50	2.597×10^{-4}	100	2.484×10^{-4} 1.064×10^{-3}	93 7	2.099×10^{-4} 8.850×10^{-4}	77 23	1.943×10^{-4} 9.246×10^{-4}	86 14

Table 10

Histogram Burst Rates Summary for THA

concentration (μM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1	2.141×10^{-4} 8.837×10^{-3}	54 46	1.481×10^{-4} 5.783×10^{-4} 1.100×10^{-2}	44 17 39	1.398×10^{-4} 9.277×10^{-4} 1.284×10^{-2}	49 18 33	2.541×10^{-4} 1.385×10^{-2}	64 36
10	1.989×10^{-4} 9.842×10^{-3}	56 44	9.774×10^{-5} 4.462×10^{-4} 1.583×10^{-2}	40 29 31	9.520×10^{-5} 7.180×10^{-4} 1.354×10^{-2}	57 28 15		
20	1.841×10^{-4} 7.479×10^{-4} 1.060×10^{-2}	59 9 32	1.702×10^{-4} 5.116×10^{-4} 1.479×10^{-2}	57 8 35	2.730×10^{-4} 1.444×10^{-2}	72 28	1.357×10^{-4} 5.552×10^{-4} 2.032×10^{-2}	48 30 22
50	2.192×10^{-4} 1.384×10^{-2}	67 33	1.963×10^{-4} 1.305×10^{-2}	67 33	1.672×10^{-4} 9.510×10^{-4} 1.341×10^{-2}	64 12 24	2.131×10^{-4} 1.832×10^{-2}	79 21

Table 11

Histogram Closing Rates Summary for THA Burst Analysis

concentration (μM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1	4.947×10^{-5} 1.356×10^{-3}	62 38	1.000×10^{-4} 1.658×10^{-3}	56 44	1.374×10^{-4} 2.199×10^{-3}	61 39	2.076×10^{-4} 2.790×10^{-3}	68 32
10	1.981×10^{-4} 1.485×10^{-3}	9 90	4.091×10^{-4} 2.527×10^{-3}	18 82	2.574×10^{-4} 3.831×10^{-3}	25 75		
20	2.310×10^{-4} 2.556×10^{-3}	10 90	3.242×10^{-4} 3.071×10^{-3}	12 88	5.247×10^{-4} 4.185×10^{-3}	18 82	2.957×10^{-4} 6.205×10^{-3}	17 83
50	1.316×10^{-4} 2.938×10^{-3}	9 91	1.823×10^{-4} 4.302×10^{-3}	10 90	1.821×10^{-4} 4.733×10^{-3}	8 92	5.970×10^{-4} 9.431×10^{-3}	18 82

Table 12

Histogram Opening Rates Summary for Physostigmine

concentration (μM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1			1.351×10^{-4} 1.664×10^{-3}	33 67	6.419×10^{-5} 1.822×10^{-3}	36 64	6.225×10^{-5} 2.546×10^{-3}	38 62
10	1.062×10^{-3}	100	1.394×10^{-4} 1.141×10^{-3}	16 84	1.692×10^{-4} 1.155×10^{-3}	17 83	7.149×10^{-5} 1.202×10^{-3}	21 79
20	9.272×10^{-4}	100	8.372×10^{-4}	100	8.374×10^{-4}	100	1.005×10^{-4} 8.878×10^{-4}	13 87
50								

Table 13

Histogram Burst Rates Summary for Physostigmine

concentration (μM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1			1.809×10^{-4} 2.329×10^{-3}	39 61	4.716×10^{-5} 4.691×10^{-4} 2.949×10^{-3}	39 14 37	5.819×10^{-5} 1.626×10^{-3} 8.120×10^{-3}	41 33 26
10	2.117×10^{-3}	100	1.141×10^{-4} 1.002×10^{-3} 6.854×10^{-3}	22 38 40	1.663×10^{-4} 9.978×10^{-4} 1.082×10^{-2}	24 34 42	6.862×10^{-5} 8.629×10^{-4} 1.242×10^{-2}	28 32 40
20	3.420×10^{-3}	100	1.559×10^{-4} 9.239×10^{-4} 1.180×10^{-2}	23 23 54	2.660×10^{-4} 1.223×10^{-3} 1.649×10^{-2}	31 16 53	1.716×10^{-4} 1.030×10^{-3} 2.370×10^{-2}	29 24 47
50	4.685×10^{-4} 1.083×10^{-2}	40 60	6.684×10^{-5} 4.498×10^{-4} 2.699×10^{-2}	39 23 38	9.466×10^{-5} 5.678×10^{-4} 3.894×10^{-2}	42 20 38	7.091×10^{-5} 6.021×10^{-4} 5.272×10^{-2}	40 19 41

Table 14

Histogram Closing Rates Summary for Physostigmine Burst Analysis

concentration (μM)	70 mV (sec)	area %	100 mV (sec)	area %	120 mV (sec)	area %	140 mV (sec)	area %
1			5.932×10^{-6} 3.263×10^{-3}	99 1	1.441×10^{-5} 4.601×10^{-3}	97 3	1.545×10^{-5} 6.580×10^{-3}	96 4
10	5.75×10^{-5} 1.67×10^{-4} 1.65×10^{-3}	24 2 74	3.686×10^{-5} 1.076×10^{-4} 2.934×10^{-3}	16 1 82	2.323×10^{-5} 3.793×10^{-4} 5.156×10^{-3}	33 3 64	2.20×10^{-5} 6.55×10^{-4} 7.45×10^{-3}	47 1 52
20	3.558×10^{-5} 6.583×10^{-4} 1.760×10^{-3}	20 1 79	1.683×10^{-5} 3.476×10^{-4} 3.720×10^{-3}	25 7 68	5.100×10^{-5} 5.385×10^{-4} 4.970×10^{-3}	11 4 85	1.651×10^{-5} 4.944×10^{-4} 7.164×10^{-3}	39 2 59
50	6.478×10^{-5} 2.816×10^{-3}	24 76	3.871×10^{-5} 3.328×10^{-4} 6.339×10^{-3}	3 9 88	4.747×10^{-5} 9.356×10^{-4} 1.048×10^{-2}	4 6 90	7.454×10^{-5} 1.512×10^{-2}	6 94

DISCUSSION

The results of the experiments collected together here revealed that the anticholinesterases THA and physostigmine interacted directly with nAChR at low concentrations. Significant effects were observed for THA and physostigmine at concentrations as low as 1 μM. These observations may be of importance in understanding the effects of anticholinesterases on both peripheral nAChR and centrally located ganglionic nAChR. The expanded effects of these drugs, by exerting an influence at sites other than AChE, may introduce complexities into their mechanisms of action that have hitherto been unobserved by less sensitive methods. The overall effects of these drugs as measured clinically may be reflected in the different efficacies observed in the reversal of curare-induced neuromuscular blockade (Wachtel, 1990) and to the various degrees of success that have been reported in the treatment of Alzheimer disease by the use of THA and physostigmine (review, Becker & Giacobini, 1988). Although there is a large body of literature on the interaction of anticholinesterases on the nAChR, there are only a handful that have utilized patch clamp methods (Wachtel, 1988, 1990; Aracava, et al., 1987; Shaw, et al., 1985). The aim of the present study has been to demonstrate the differences in nAChR channel behavior in the presence of THA and physostigmine and to compare them to a pure channel blocker about which much is known, the local anesthetic, QX-222, as well as to each other. There are several significant differences that will be discussed.

Anticholinesterase Binding to nAChR

Local anesthetics have not been shown to inhibit the binding of cholinergic agonists to nAChR. This is consistent with the present conclusion that QX-222 does not interfere with the binding of ^{125}I-BGT to solubilized muscle nAChR. There are examples

of anticholinesterases that inhibit ligand binding at mammalian ganglionic nAChR (Mo, Dun, & Karczmar, 1985), ganglionic nAChR from Aplysia (Carpenter, Greene, Shain, & Vogel, 1976), nAChR from Torpedo electric organ (Bakry, et al., 1982), and muscarinic AChR (Misgeld, Muller, & Polder, 1989). Physostigmine could not be shown to interfere with the binding of either ^{125}I-BGT to solubilized receptor or to polyclonal antibodies directed against the nAChR. However, THA specifically inhibited both species of ligand. Both THA and physostigmine exhibited similar pharmacologies as measured by $^{22}Na^{+}$ influx into PC12 cells, a cell line that expresses a ganglionic nAChR. Neither drug could be shown to possess agonist properties. Preincubation of PC12 cells in the presence of THA or physostigmine resulted in significant attenuation in carbamoylcholine-induced flux of $^{22}Na^{+}$. This reductioin in ion flux was independent of the preincubation time. Each drug elicited a curious response to either coincubation of the drugs with the PC12 cells in the presence of agonist during the fluxing procedure or preincubation of the cells with either drug before flux stimulation. The influx was similarly inhibited under each condition. This indicates that the receptors may rapidly undergo a step towards desensitization during preincubation with the anticholinesterases. Virtually no anticholinesterase was present during the flux procedure after preincubation, therefore, no anticholinergic effect would be seen on the influx of ions through the nAChR if these drugs acted as simple channel blockers since all blocking drug would have diffused away. Instead, there may be a conformational lag between diffusion of the drugs and the activation of the channels. Preincubation of nAChR in the presence of concentrations of agonists that were too low to activate receptors were reported to place the receptors in a desensitizable state (Fiekers, Neel, & Parsons, 1987). Such a mechanism may be postulated to have occurred when the cells were preincubated with anticholinesterases. Although physostigmine did not interfere with BGT binding, it may exert its effects at a location other than the BGT-binding site. Therefore, the active site at which physostigmine binds might also be the same site at which THA binds but would be

undetectable by BGT binding experiments. Before activation, the receptors are not inactive but become desensitized upon binding of agonist during the activation step.

The conformational changes during the recovery of the nAChR after exposure to THA or physostigmine were significantly different. Physostigmine did not induce a lasting effect on the inhibition of influx, and the cells recovered quickly after the removal of physostigmine from the cells. In Figure 6, the recovery after physostigmine preincubation revealed what appeared to be a simple diffusion away from the nAChR by the anticholinesterase. The time of flux recovery after preincubation with THA exhibited a sigmoidal relationship indicating a more complex interaction between nAChR and anticholinesterase. It is possible that THA binds specifically to more than one site, and its removal is of a cooperative nature. While the inhibition of flux was similar for both anticholinesterases, these differences suggested a role of specific binding sites for THA and physostigmine that underlie the differences in their kinetic relationships with ganglionic nAChR.

One might predict that if these anticholinesterases had extra-channel sites of action, then, by competition binding experiments using the proper ligands, they would inhibit the binding of those ligands to nAChR. This may have been proved by the inhibition of the binding of ^{125}I-BGT to solubilized nAChR by THA although physostigmine could not be shown to exhibit similar inhibition. Physostigmine has been shown in other systems, using non-solubilized nAChR in Torpedo membranes, to inhibit the binding of ^{125}I-BGT and [^{3}H]H_{12}-histrionicotoxin (Sherby, et al., 1985) and the binding of ^{125}I-BGT to solubilized nAChR in homogenates from Aplysia (Carpenter, et al., 1976).

Other investigators have also found that physostigmine and THA differed in their effects on ligand binding. The ability of physostigmine and THA to inhibit the binding of [^{3}H]-nicotine to human cortical slices were compared by Perry et al. (1988a). In that study it was found that physostigmine was much less potent in inhibiting the binding of nicotine to cortical nAChR and mAChR. The IC_{50} values for THA and physostigmine as measured

by their ability to displace [^{3}H]-nicotine binding were 2 x 10^{-5} M and 2 x 10^{-3} M, respectively, a difference of a thousand-fold. The authors proposed that THA may exert its therapeutic effects independently of its anticholinesterase effects at low concentrations by regulating receptor-mediated feedback mechanisms of ACh release but that physostigmine acted at concentrations that would be of no therapeutic value (Perry, Smith, Xuereb, Keith, & Perry, 1988b). Since both THA and physostigmine were clearly capable of inducing channel blockade and only THA inhibited the binding of ^{125}I-BGT to nAChR, their site(s) of action may overlap but may not be located near the binding sites for ACh and BGT. These studies, however, cannot rule out the nAChR pore as one possible site of action of anticholinesterases.

Single-Channel Kinetics of nAChR and Anticholinesterase Interactions

The actions of QX-222 upon nAChR have been well-characterized previously by other investigators (Neher & Steinbach, 1978; Neher, 1983; Charnet, Labarca, Leonard, Vogelaar, Czyzyk, Gouin, et al., 1990; Leonard, Charnet, Labarca, Vogelaar, Czyzyk, Gouin, et al., 1991). The use of QX-222 in this study was to justify the data from our patch clamp apparatus and to serve as a model against which to compare the data found using THA and physostigmine. The original patch clamp study by Neher and Steinbach (1978) observed the effects of QX-222 on frog sartorius muscle preparations while the system in this study involved a clonal cell line that expresses nAChR. Therefore, disagreement between the values given in the original studies versus the present ones may be attributed in part to differences in equipment and biological preparation. A second reason may also be that in the present study, potassium was the predominant permeant ion while in the original study it was sodium. Inversion of the relative extracellular concentrations of potassium and sodium ions depolarized the cell preparations so that variability of the membrane potential as a factor in our studies became a minimal consideration. The predictions of single channel behavior in the presence of a simple channel blocker should have been borne out no matter the cell system or ionic species,

however, and this proved to be the case. As an example, the easiest data comparisons can be done with channel open time and its voltage dependence. In the original studies, a plot of mean channel open times versus pipette holding potential revealed a channel closing constant, α, that was strongly dependent upon voltage with an e-fold change for every 83 mV change in potential. In the present study, that same relationship was preserved, albeit by a slightly different magnitude, with an e-fold change in α for every 113 mV change in holding potential.

Predictions from the linear sequential blocking model, proposed by Ruff (1977) and validated by Neher and Steinbach (1978) for QX-222, include the following effects of channel blocking drugs: 1) Channel open time must be shortened; 2) longer bursts of brief openings; 3) single channel current amplitude should not be altered; 4) channel open time decreases with increasing drug concentration; 5) burst duration is prolonged by increasing drug concentration; and 6) the duration of brief blocking events within a burst is independent of drug concentration. The data from the present study nearly fit all these criteria.

The aim of the present study was to compare the effects of THA and physostigmine on single channel nAChR behavior to each other as well as to QX-222. Physostigmine and THA certainly are channel blockers. In the greatest part, both anticholinesterase inhibitors resembled QX-222, and, therefore, at least in part, the mechanisms of actions of these two classes of drugs may be similar. Physostigmine, like THA and QX-222, demonstrated no agonist-like properties, and at high concentrations channel activity was almost totally shut down by concentrations just higher than 50 μM. These two observations were in sharp contrast to those observations made by other investigators using another biological preparation (Shaw, et al., 1985).

Shaw et al. (1985) found that the channel activity recorded from frog (Rana pipiens) muscle fibers could be observed in the presence of physostigmine concentrations as high as 600 μM and that 0.5 μM physostigmine possessed agonist activity not unlike

that of ACh. In this study, no anticholinesterase was found to have agonist properties. In fact, a patch pipette containing no agonist but only recording solution showed good channel activity. The mean channel open time at 100 mV was 950 ms compared 2.3 ms for control patches containing 100 nM ACh. The opening rates were bimodally distributed with rate constants of 1.57×10^{-4} and 1.68×10^{-3} for τ_f and τ_s, respectively. Compared to channels observed under control conditions in the presence of 100 nM ACh and 100 mV (Table 5), the slow component of liganded channels was approximately 40% slower than the slow component of unliganded channels. The fast component of the unliganded channels was faster by 30% compared to those of liganded channels. However, the percent distribution of the slow and fast components for the unliganded channels was 40% and 60%, respectively, whereas the percent distribution for the liganded channels was 67% and 33%, respectively. Bursts of activity containing very brief closures was observed for both control and unliganded channels. The control ACh concentration of 100 nM would indicate that many open receptors probably contained only one bound agonist molecule although two components could be definitely seen. Two components were also seen in the presence of very high concentrations of agonists. Other authors have concluded that these phenomena support the theory that brief closures within a burst are not the result of singly-occupied receptors (Sine & Steinbach, 1988) since they occur in both singly- and doubly-occupied states. Data from unliganded channel records indicate that brief closures may not represent the status of receptor-ligand occupancy. These data support the concept that agonists increase the probability of opening a channel by destabilizing the closed channel (Jackson, 1986) since the distributions of slow opening components became reversed and predominated in the liganded state. The binding of a second agonist molecule more strongly destabilizes the closed state than the binding of one agonist molecule, and thus one expects to see a shift to a larger proportion of the slow opening components in the presence of higher concentrations of agonists (Jackson, 1988). We propose that channels are constantly and periodically opening and closing in the unliganded state. The presence

of a molecule, such as an anticholinesterase, within the channel pore of an AChR may not be necessary in order to observe brief closures within a burst of openings. However, the closures within a burst in the presence of an anticholinesterase were observed to remain closed for a longer period of time compared to those within control patches in the presence of ACh. Anticholinesterases may impede the action of agonists by interacting with the AChR at sites that partially offset the ability of agonists to destabilize the closed state. One would observe, therefore, a prolonged closed state which would be consistent with the observations in this study. The possibility of binding sites outside those of the pore and those for ACh and BGT have been determined by the experiments detailed here and in other studies using α-dendrotoxin, a long, snake venom neurotoxin (Conti-Tronconi & Raftery, 1986). These authors have proposed that there may be several non-equivalent ligand binding sites on the nAChR and that separate binding sites could be responsible for activation and desensitization as parallel but independent processes (Dunn, Conti-Tronconi, & Raftery, 1983).

Qualitatively, THA and physostigmine resembled the actions of QX-222 in that, as predicted, the mean channel burst length was considerably lengthened. This parameter was an important measurement in that the charge carried through the open channel in the presence of an anticholinesterase must have been significantly increased compared to open channels in control patches since, after subtratcting the closed time within a burst from the total open times, the mean channel open times for a burst in the presence of an anticholinesterase were still greater than control patches, a fact not predicted by the sequential channel blocking model (Neher & Steinbach, 1978). Lengthening the duration of the mean channel open time increases the charge carried through the nAChR, but the increased temporal aspect of the event decreases the charge density around the channel. The result may be a lengthened time constant of the end-plate potential as measured on in vitro preparations (Scuka, 1987). Physostigmine increased the mean burst length 3-fold over the maximum observed for THA and about 9-fold over the maximum observed with

QX-222. This was in contrast to studies by Shaw et al. (1985) in which physostigmine was not observed to increase the burst length. At 1 μM, physostigmine did not appreciably lengthen the burst but THA increased it markedly by 2.7-fold at 70 mV. In fact, it was the only drug to do so at this concentration. This may be a significant point in discriminating the physiological effects between physostigmine and THA since low concentrations are more physiologically relevant.

The most profound difference between THA, physostigmine, and QX-222 was that of a concentration-dependent effect upon the mean channel block time within a burst. Based on the linear sequential channel blocking model, the mean block time should be independent of the concentration of a channel blocking drug (Neher & Steinbach, 1978). Instead, the duration of the block time should be dependent upon the voltage across the cell membrane. Both anticholinesterases and QX-222 exhibited the predicted voltage dependence of the channel blocking rate. THA and physostigmine, and perhaps QX-222, demonstrated a significant positive correlation between the concentration of drug present and the mean block time. This effect was qualitatively similar to the single-channel effects observed for nAChR in the presence of the inhalational anesthetic, isoflurane (Brett, Dilger, & Yland, 1988) although isoflurane also reduced the mean burst length in the same study. The forward rate of channel blocking for physostigmine was at least as sensitive as QX-222 to membrane voltage changes. THA was much less sensitive by half to changes in membrane potential as compared to QX-222 and physostigmine. This additional effect, not predicted by the linear sequential model of channel blocking, may be the result of sites of actions by THA and physostigmine that are outside the channel.

The observation that the rate of channel unblocking by THA and physostigmine was concentration-dependent may be explained if a state of desensitization accompanied the blocked state. One would predict that if this were the case, the desensitized state might persist after THA or physostigmine had already diffused away. This would be consistent with the observation that $^{22}Na^{+}$ influx was inhibited by preincubating PC12 cells with

anticholinesterases before activation and that channel activity upon subsequent exposure to agonist was diminished. As has been noted, other authors have observed this phenomenon using low concentrations of agonists in similar experiments (Boyd, 1987). The low apparent K_d of THA and physostigmine as measured by the patch clamp data may reflect either the actual affinity of these molecules for the nAChR or they may be artificially lowered by the prolongation of channel blockade, since the rate of channel unblocking is one parameter used in the measurement of the K_d. Both physostigmine and THA have high rates of association with the nAChR at low drug concentrations. In clinical terms, this may be of importance in that both drugs may have direct effects upon nAChR at physiologic concentrations.

The differences between physostigmine and THA that may contribute to their pharmacological efficacies may lie in their different effects at very low concentrations. The main example of this is the ability of THA to increase the burst length significantly at 1 mM compared with no effect at the same concentration of physostigmine. The K_d values for both drugs were similar as measured by patch clamp and flux experiments. However, THA had an additional effect upon ion flux in that after preincubation of the PC12 cells with a low concentration of THA, there was a significant delay in flux recovery that proceded with a sigmoidal time course not seen with physostigmine. These observations may be consistent with the hypothesis that THA stabilizes the nAChR in a desensitizable or a desensitized state that occurs at lower concentrations than physostigmine.

These direct effects upon the nAChR may antagonize the effects inhibition of AChE might have upon synaptic transmission characteristics. Anticholinesterases lengthen the time constant and increase the amplitude of the synaptic epp. This prevents repeated synaptic transmission since the epp would not have been reduced below the threshold for an action potential before the next pre-synaptic depolarization arrived. This inhibition can be reversed by a low concentration of curare (Peper, Sterz, & Bradley, 1981; Bradley, Pagala, & Edge, 1986a). Curare blocks nAChR and reduces the amplitude of the epp and

shortens its time constant so that presynaptic stimulation results in functioning at a more normal frequency. Physostigmine and THA have been tested for use in AD with the rationale that by making the ACh more readily available, synaptic functioning would be restored. However, increasing synaptic ACh too greatly may not only have no improving effect but may result in inhibition of synaptic transmission (Bradley, et al., 1988). The concomitant channel blocking actions of physostigmine and THA with their anticholinesterase may promote their facilitatory effects in neuronal functioning, thus improving cholinergic mechanisms in the brains of AD victims. High concentrations of anticholinesterase have been noted to reverse the effects of anticholinesterase poisoning upon preparations of rat extensor digitorum longus muscle and nerve, presumably by a channel blocking effect (Bradley, unpublished observation). This channel-blocking effect may also be responsible for the different efficacies observed in the ability of anticholinesterases to reverse neuromuscular blockade induced by tubocurare after major surgery.

Anticholinesterases and Desensitization of the nAChR

At low concentrations of QX-222, Neher and Steinbach (1978) determined that blockade of the nAChR occurred in the open state of the channel. Other substances also block the nAChR, and models of channel blockade by physically plugging the open channel pore have been devised (Adams, 1976; Manalis, 1977). However, there is no qualitatively obvious difference in the blocked state and the non-conducting state. Models of the subconducting nAChR using curare have implied that there is a site on the nAChR that is accessible via either the closed or open receptor (Strecker & Jackson, 1989), unlike the site for local anesthetics that must occur around the open nAChR. An extension of the subconducting nAChR model would be one for the non-conducting nAChR in which the receptor pore is not physically blocked but is rendered non-conducting by allosteric mechanisms, such as is proposed by the extended channel model (Dilger & Brett, 1991). In this model, the channel may close before the dissociation of the bound drug. This

model was used to explain the observation that general anesthetics, enflurane and isoflurane, decrease the burst length of nAChR as determined by single-channel techniques (Dilger & Brett, 1991). Although anticholinesterases increase the burst length of nAChR, this model may still be useful in explaining the concentration-dependent increase in the mean channel block time observed in the present study. The mean channel block time may have increased in the presence of anticholinesterases due to their ability to rapidly, briefly, and allosterically induce a non-conducting state of the nAChR and that state is not qualitatively distinguishable from the blocked state induced by local anesthetics. This would also not be inconsistent with the parallel model of channel blocking, a model defined to explain the slow unblocking rates of tetracaine, phencyclidine, and N-allylnormetazocine, in which the opening and closing of the nAChR channel can occur independently of the presence of a blocker (Papke & Oswald, 1989).

Introducing a brief desensitized state of the receptor may give a similar appearance in the patch clamp data as a classical blocker, namely that of brief closings within a burst of openings. However, the patch clamp data combined with the data from flux studies may point to a more complicated interaction between nAChR and anticholinesterases. The total open time per burst in the presence of these anticholinesterases increased markedly compared to that of QX-222. This phenomenon was strongly voltage-dependent as was the duration of the blocking events within bursts. Other investigators have concluded that the time-course of desensitization of nAChR is voltage dependent (Magleby & Pallotta, 1981). Dilger and Brett (1990) have determined that nAChR in the presence of 1 μM ACh (and up to 1000 μM) demonstrated significant and rapid desensitization with onset time constants varying around 100 ms. However, they saw no voltage-dependence at 1 μM ACh. In the present study, the presence of an anticholiesterase led to closings within bursts that were not unlike those of control patches except in their number and duration. We propose that anticholinesterases hasten the onset and lengthen the duration of desensitization such that the channel closes briefly and without re-entering the opeing state,

consistent with an extended channel model. Although this phenomenon may not properly be called 'desensitization,' its properties are consistent with the non-conducting state which is qualitatively identical. The closings within bursts may therefore represent voltage-dependent allosteric modulation of channel closing with or without the presence of agonist.

Bursting behavior of the nAChR is important in the behavior of junctional transmission. This increased charge transfer has a direct effect upon the height of the epp. Since cholinergic receptors are scarce in the basal nuclei of AD victims, this phenomenon may serve to heighten the effectiveness of each channel remaining. The probability of the heightened post-synaptic potential (PSP) to attain the threshold for synaptic transmission would be increased. It may therefore be that anticholinesterases help to redistribute the charge transfer in restoring synaptic functioning in AD neurons by altering channel kinetics. By inhibiting AChE, the probability that a nAChR would open is increased while the concomitant intermittent channel blockade increases the mean open time per burst of a receptor. The increased PSP would result in restored neurotransmission if the threshold for transmission is reached.

Physostigmine and THA differed in their distributions of fast and slow opening rates during bursts, as compared in Tables 10 and 13. Unlike QX-222, both drugs introduced an intermediate time constant. However, the fast component occurred at a lower membrane potential for THA, and at 100 mV and 1 μM the slow component comprised only 39% of the total rates while for physostigmine under the same conditions the slow component accounted for 61%. The faster components increase in proportion at higher concentrations and membrane potentials. This would be expected since the forward rates of blocking were voltage and concentration dependent. That difference under physiological conditions may be important in understanding the different therapeutic efficacies of these drugs.

Outlook. Treatment strategies in AD have remained focused on the cholinergic hypothesis (Perry, 1986, 1988). The efficacy of treatment of AD with THA is still largely unknown (Boller & Forette, 1989), and THA remains an investigational drug (U.S. Food and Drug Administration, 1992). THA remains, however, the best choice in the search for a treatment of the cognitive deficits caused by AD, and positive treatment outcomes are still being reported (Levy, 1990). Differences in anticholinesterases used in the treatment of Alzheimer disease have begun to focus on areas of metabolism such as their effects on ACh release, synthesis, and the HACU (Hallak & Gizcobini, 1989). New analogs of physostigmine (Marta, et al., 1988; Brufani, Castellano, Marta, Murroni, Oliverio, Pagella, et al., 1988) and THA (Shutske, et al., 1988) have been introduced. In the future, professionals researching anticholinesterases for the treatment of AD should consider not only the availability and potency of these drugs as anticholinesterases, but also their direct effects on nAChR.

SUMMARY

Physostigmine and THA have direct effects upon both ganglionic and muscle type nAChR. Solubilized preparations of nAChR from an established muscle cell line, TE-671, revealed that low concentrations of THA inhibited the binding of a specific ligand, ^{125}I-BGT. Physostigmine was not shown to inhibit the binding of ^{125}I-BGT in this system. The sites to which THA bound may not be necessarily located at or near the BGT binding site since THA was shown to inhibit the binding of polyclonal antibodies from the sera of myasthenic patients to nAChR extracted from TE-671 cells or from human foot muscle. Physostigmine could not be demonstrated to inhibit the binding of antibody to solubilized receptor.

The actions of physostigmine and THA upon ganglionic nAChR were studied in preparations of cultured PC12 cells. These drugs caused a block of sodium influx at micromolar concentrations with IC_{50} values around 15 μM to 25 μM for both drugs. Blockade occurred regardless of the preincubation of these drugs with PC12 cells or the coincubation during activation. The inhibition of flux proceded similarly for both drugs but flux recovery after preincubation with THA lagged behind that for physostigmine. The shape of the recovery curve for THA indicated at least a biphasic response whereas that for physostigmine was a simple, rapid dissociation. Therefore, the effects of THA are longer-lived than those of physostigmine or THA may have the additional effect of desensitizing nAChR .

The data in these studies support the hypothesis that physostigmine and THA are channel blockers. Compared to a classical charged channel blocker, QX-222, these drugs behaved very similarly. Both physostigmine and THA increased the mean burst length of

channel opening by blocking the open nAChR channel. Although physostigmine increased burst length by the largest margin, only THA increased burst length at 1 μM compared to no effect for channels in the presence of 1 μM physostigmine. The effect at such a low concentration was of such magnitude as possibly to be clinically relevant. These drugs reversed the effect of hyperpolarization on mean channel open time by markedly shortening this parameter. The forward rate of channel blocking was strongly voltage dependent for physostigmine whereas it showed less sensitivity to changes in membrane potential with THA. Physostigmine and THA demonstrated an unusual property not predicted by the linear sequential blocking model in that the rate of channel unblocking was dependent upon the concentration of the drug present. This implied that these drugs may influence channel behavior at sites outside the receptor pore. Possibly, these phenomena could be explained by a mechanism such as desensitization of the receptor or a mechanism qualitatively similar to desensitization but resulting in a non-conducting channel by allosteric modulation. The unblocking rate constant was voltage dependent and increased with decreasing membrane potential for both drugs as well as QX-222. The unblocking rate constant was also concentration-dependent for THA and physostigmine in that the lower the concentration, the higher the rate of unblocking. The resulting apparent K_d between nAChR and anticholinesterases was then dependent upon membrane voltage as well as the forward and backward rate constants of channel blocking. The results could be explained qualitatively by a model of channel blocking not unlike the extended model of channel block whereby the nAChR is allowed to close while the drug is still bound. The K_d measured for THA and physostigmine covered only a narrow range between 1 μM and about 15 μM for both drugs, but the low values of association were surprising and may be phyiologically relevant. These findings may provide evidence that physostigmine and THA induce a state of brief desensitization of the nAChR or place the receptor into a desensitizable state leading into a state of desensitization upon the binding of an agonist. The site of action for

these drugs in inducing this phenomenon may not be confined to the nAChR channel, but they may bind elsewhere on the receptor and exert their effects allosterically.

REFERENCES

Adams, P. R. (1976). Drug blockade of open endplate channels. Journal of Physiology (London), 260, 531-552.

Akaike, A., Ikeda, S. R., Brookes, N., Pascuzzo, G. J., Rickett, D. L., & Albuquerque, E. X. (1984). The nature of the interactions of pyridostigmine with nicotinic acetylcholine receptor-ionic channel complex. II. Patch clamp studies. Molecular Pharmacology, 25, 102-112.

Alzheimer, A. (1977). A unique illness involving the cerebral cortex. A case report from the mental institution in Frankfurt am Main. In D. A. Rottenberg & F. H. Hochberg (Eds.), C. N. Hochberg & F. H. Hochberg (Trans.), Neurological classics in modern translation (pp. 41-43). New York: Hafner Press. (Reprinted from Allgemeine Zeitschrift für Psychiatrie und Psychisch-Gerichtliche Medizin, 1907, 64, 146-148)

Aracava, Y., Deshpande, S. S., Rickett, D. L., Brossi, A., Schönenberger, B., & Albuquerque, E. X. (1987). The molecular basis of anticholinesterase actions on nicotinic and glutamatergic synapses. In D. B. Drachman (Ed.), Myasthenia gravis: Biology and treatment (pp. 226-255). New York: The New York Academy of Sciences.

Bakry, N. B., Eldefrawi, A. T., Eldefrawi, M. E., & Riker, W. F. (1982). Interactions of quaternary ammonium drugs with acetylcholinesterase and acetylcholine receptor of Torpedo electric organ. Molecular Pharmacology, 22, 63-71.

Ball, M. J. (1977). Neuronal loss, neurofibrillary tangles and granulovascuolar degeneration in the hippocampus with ageing and dementia. Acta Neuropathologica (Berlin), 37, 111-118.

Ball, M. J. (1980). Features of Creutzfeldt-Jakob disease in brains of patients with familial dementia of Alzheimer type. Canadian Journal of Neurological Science, 7, 51-57.

Bartus, R. T. (1989). The impending Alzheimer's crisis: Can we be saved by the revolution? Neurobiology of Aging, 10, 383-384.

Bartus, R. T., Dean, R. L., & Beer, B. (1982). The cholinergic hypothesis of geriatric memory dysfunction. Science, 217, 408-417.

Becker, R. E., & Giacobini, E. (1988). Mechanisms of cholinesterase inhibition in senile dementia of the Alzheimer type: Clinical, pharmacological, and therapeutic aspects. Drug Development Research, 12, 163-195.

Blessed, G., Tomlinson, B. E., & Roth, M. (1968). The association between quantitative measures of dementia and of senile change in the cerebral grey matter elderly subjects. British Journal of Psychiatry, 114, 797-811.

Boller, F., & Forette, F. (1989). Alzheimer's disease and THA: A review of the cholinergic theory and of preliminary results. Biomedicine and Pharmacotherapy, 43, 487-491.

Boller, F., Mizutani, T., Roessmann, U., & Gambetti, P. (1980). Parkinson disease, dementia, and Alzheimer disease: Clinicopathological correlations. Annals of Neurology, 7, 329-335.

Bowen, D. M., Allen, S. J., Benton, J. S., Goodhardt, M. J., Haan, E. A., Palmer, A. M., Sims, N. R., Smith, C. C. T., Spillane, J. A., Esiri, M. M., Neary, D., Snowdon, J. S., Wilcock, G. K., & Davison, A. N. (1983). Biochemical assessment of serotonergic and cholinergic dysfunction and cerebral atrophy in Alzheimer's disease. Journal of Neurochemistry, 41, 266-272.

Bowen, D. M., Smith, C. B., White, P., & Davison, A. N. (1976). Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. Brain, 99, 459-496.

Boyd, N. D. (1987). Two distinct kinetic phases of desensitization of acetylcholine receptors of clonal rat PC12 cells. Journal of Physiology (London), 389, 45-67.

Bradley, R. J., Edge, M. T., Moran, S. G., & Freeman, A. M. (1988). Effects of cholinergic drugs used in Alzheimer therapy at the mammalian neuromuscular junction. In R. Becker & E. Giacobini (Eds.), Current research in Alzheimer therapy (pp. 100-209). New York: Taylor and Francis.

Bradley, R. J., Pagala, M. K. D., & Edge, M. T. (1986a). Curare can reverse the failure in muscle contraction caused by an AChE inhibitor. Brain Research, 377, 194-198.

Bradley, R. J., Sterz, R., & Peper, K. (1986b). Agonist and inhibitory effects of pyridostigmine at the neuromuscular junction. Brain Research, 376, 199-203.

Brett, R. S., Dilger, J. P., & Yland, K. F. (1988). Isoflurane causes "flickering" of the acetylcholine receptor channel: Observations using the patch clamp. Anesthesiology, 69, 161-170.

Brufani, M., Castellano, C., Marta, M., Murroni, F., Oliverio, A., Pagella, P. G., Pavone, F., Pomponi, M., & Rugarli, P. L. (1988). From physostigmine to physostigmine derivatives as new inhibitors of cholinesterase. In E. Giacobini & R. Becker (Eds.), Current research in Alzheimer therapy (pp. 343-352). New York: Taylor and Francis.

Buyukuysal, R. L., & Wurtman, R. J. (1989). Tetrahydroaminoacridine but not 4-aminopyridine inhibits high-affinity choline uptake in striatal and hippocampal synaptosomes. Brain Research, 482, 371-375.

Caine, E. D. (1980). Cholinomimetic tratment fails to improve memory disorders. New England Journal of Medicine, 303, 585-586.

Carpenter, D. O., Greene, L. A., Shain, W., & Vogel, Z. (1976). Effects of eserine and neostigmine on the interaction of α-bungarotoxin with Aplysia acetylcholine receptors. Molecular Pharmacology, 12, 999-1006.

Charnet, P., Labarca, C., Leonard, R. J., Vogelaar, N. J., Czyzyk, L., Gouin, A., Davidson, N., & Lester, H. A. (1990). An open-channel blocker interacts with adjacent turns of α-helices in the nicotinic acetylcholine receptor. Neuron, 2, 87-95.

Chini, B., & Clementi, F. (1989). Nicotinic acetylcholine receptors in the nervous system: Structure, distribution and modification in brain pathology. Neurobiology of Aging, 10, 371-374.

Colquhoun, D., & Sakman, B. (1985). Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. Journal of Physiology (London), 369, 501-557.

Colquhoun, D., & Sigworth, F. J. (1983). Fitting and statistical analysis of single channel records. In B. Sakman & E. Neher (Eds.), Single-channel recording New York: Plenum Press.

Conti-Tronconi, B. M., & Raftery, M. A. (1986). Nicotinic acetylcholine receptor contains multiple binding sites: Evidence from binding of α-dendrotoxin. Proceedings of the National Academy of Science, USA, 83, 6646-6650.

Coyle, J. T., Price, D. L., & DeLong, M. R. (1983). Alzheimer's disease: A disorder of cortical cholinergic innervation. Science, 219, 1184-1190.

Cross, A. J., Crow, T. J., Ferrier, I. N., Johnson, J. A., Bloom, S. R., & Corsellis, J. A. N. (1984). Serotonin receptor changes in dementia of the Alzheimer type. Journal of Neurochemistry, 43, 1574-1581.

Cross, A. J., Crow, T. J., Johnson, J. A., Joseph, M. H., Perry, E. K., Perry, R. H., Blessed, G., & Tomlinson, B. E. (1983). Monamine metabolism in senile dementia of Alzheimer type. Journal of the Neurological Sciences, 60, 383-392.

Crow, T. J., Cross, A. J., Cooper, S. J., Deakin, J. F. W., Ferrier, I. N., Johnson, S. A., Joseph, M. H., Owen, F., Poulter, M., Lofthouse, R., Corsellis, J. A. N., Chambers, D. R., Blessed, G., Perry, E. K., Perry, R. H., & Tomlinson, B. E. (1984). Neurotransmitter receptors and monoamine metabolites in the brains of patients with Alzheimer-type dementia and depression, and suicides. Neuropharmacology, 23, 1561-5169.

Damasio, A. R. (1992). Alzheimer's disease and related dementias. In J. B. Wyngaarden, L. H. Smith, & J. C. Bennett (Eds.), Cecil textbook of medicine (pp. 2075-2079). Philadelphia, PA: W.B. Saunders company.

Davies, P., & Maloney, A. J. F. (1976). Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet, 2, 1403.

Davis, K. L., Hollander, E., Davidson, M., Davis, B. M., Mohs, R. C., & Horvath, T. B. (1987). Induction of depression with oxotremorine in patients with Alzheimer's disease. American Journal of Psychiatry, 144, 468-471.

Dilger, J. P., & Brett, R. S. (1990). Direct measurement of the concentration- and time-dependent open probability of the nicotinic acetylcholine receptor channel. Biophysical Journal, 57, 723-731.

Dilger, J. P., & Brett, R. S. (1991). Actions of volatile anesthetics and alcohols on cholinergic receptor channels. In E. Rubin, K. W. Miller, & S. H. Roth (Eds.), Molecular and Cellular Mechanisms of Alcohol and Anesthetics (pp. 616-627). New York: The New York Academy of Sciences.

Drachman, D. A. (1977). Memory and cognitive function in man: Does the cholinergic system have a specific role? Neurology (Minneapolis), 27, 783-790.

Drachman, D. A., & Leavitt, J. L. (1974). Human memory and the cholinergic system: A relationship to aging? Archives of Neurology, 30, 113-121.

Drukarch, B., Kits, K. S., Leysen, J. E., Schepens, E., & Stoof, J. C. (1989). Restricted usefulness of tetraethylammonium and 4-aminopyridine for the characterization of receptor-operated K^+ channels. British Journal of Pharmacology, 98, 113-118.

Dunn, S. M. J., Conti-Tronconi, B. M., & Raftery, M. A. (1983). Separate sites of low and high affinity for agonists on Torpedo californica acetylcholine receptor. Biochem., 22, 2512-2518.

Edge, M. T., Kemp, G. E., & Bradley, R. J. (1988). Low concentrations of tetrahydroaminoacridine and physostigmine block sodium flux in PC12 cells. Neuroscience Abstracts, 14, 230.

Elinder, F., Mohammed, A. K., Winblad, B., & Århem, P. (1989). Effects of THA on ionic currents in myelinated axons of Xenopus laevis. European Journal of Pharmacology, 164, 599-602.

Ellman, G., Courtney, D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7, 88-95.

Evans, D. A., Funkenstein, H., Albert, M. S., Scherr, P. A., Cook, N. R., Chown, M. J., Hebert, L. E., Hennekens, C. H., & Taylor, J. O. (1989). Prevalence of Alzheimer's Disease in a community population of older persons: Higher than previously reported. Journal of the American Medical Association, 262, 2551-2556.

Fiekers, J. F., Neel, D. S., & Parsons, R. L. (1987). Acceleration of desensitization by agonist pre-treatment in the snake. Journal of Physiology (London), 391, 109-124.

Gauthier, S., Masson, H., Gauthier, L., Bouchard, R., Collier, B., Bacher, Y., Bailey, R., Becker, R., Bergman, H., Charbonneau, R., Dastoor, D., Gayton, D., Kennedy, J., Kissel, C., Krieger, M., Kushnir, S., Lamontagne, A., St-Martin, M., Morin, J., Nair, N. P. V., Neirinck, L., Ratner, J., Suissa, S., Tesfaye, Y., & Vida, S. (1988). Tetrahydroaminoacridine and lecithin in Alzheimer's disease. In R. Becker & E. Giacobini (Eds.), Current research in Alzheimer therapy (pp. 237-245). New York: Taylor and Francis.

Greene, L. A., & Tishler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Preceedings of the National Academy of Sciences, USA, 73, 2424-2428.

Hallak, M., & Gizcobini, E. (1989). Physostigmine, tacrine and metrifonate: The effect of multiple doses on acetylcholine metabolism in rat brain. Neuropharmacology, 28, 199-206.

Hamill, O. P., Marty, A., Neher, E., Sakman, B., & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Archiv, 391, 85-100.

Harbaugh, R. E., Roberts, D. W., Coombs, D. W., Saunders, R. L., & Reeder, T. M. (1984). Preliminary Report: Intracranial cholinergic drug infusion in patients with Alzheimer's disease. Neurosurgery, 15, 514-517.

Harvey, A. L., & Rowan, E. G. (1988). Actions of THA, 3,4-diaminopyridine, physostigmine and galanthamine on neuronal K^+ currents at a cholinergic nerve terminal. In E. Giacobini & R. Becker (Eds.), Current research in Alzheimer therapy (pp. 191-197). New York: Taylor and Francis.

Henderson, V. W., & Finch, C. E. (1989). The neurobiology of Alzheimer's disease. Journal of Neurosurgery, 70, 335-353.

Hodgson, A. J., & Chubb, I. W. (1983). Isolation of the secretory form of soluble acetylcholinesterase by using affinity chromatography on edrophonium-Sepharose. Journal of Neurochemistry, 41, 654-662.

Hunter, A. J., Murray, T. K., Jones, J. A., Cross, A. J., & Green, A. R. (1989). The cholinergic pharmacology of tetrahydroaminoacridine in vivo and in vitro. British Journal of Pharmacology, 98, 79-86.

Jackson, M. B. (1986). Kinetics of unliganded acetylcholine receptor channel gating. Biophysical Journal, 49, 663-672.

Jackson, M. B. (1988). Dependence of acetylcholine receptor channel kinetics on agonist concentration in cultured mouse muscle fibers. Journal of Physiology (London), 397, 555-583.

Kellar, K. J., Whitehouse, P. J., Martino-Barrows, A. M., Marcus, K., & Price, D. L. (1987). Muscarinic and nicotinic cholinergic binding sites in Alzheimer's disease cerebral cortex. Brain Research, 436, 62-68.

Kemp, G. E., & Edge, M. T. (1987). Cholinergic function and α-bungarotoxin binding in PC12 cells. Molecular Pharmacology, 32, 356-363.

Kidd, M. (1963). Paired helical filaments in electron microscopy of Alzheimer's disease. Nature, 197, 192-193.

Kidd, M. (1964). Alzheimer's disease-an electron microscopical study. Brain, 87, 307-320.

Krnjevick, K., & Reinhardt, W. (1980). Choline excites cortical neurons. Science, 206, 1321-1323.

Kumar, V., Murphy, J., Sherman, K. A., Ashford, J. W., & Markwell, J. (1988). Cognitive and neuroendocrine changes with physostigmine in Alzheimer's disease. In R. Becker & E. Giacobini (Eds.), Current research in Alzheimer therapy (pp. 163-171). New York: Taylor and Francis.

Leonard, R. J., Charnet, P., Labarca, C., Vogelaar, N. J., Czyzyk, L., Gouin, A., Davidson, N., & Lester, H. A. (1991). Reverse pharmacology of the nicotinic acetylcholine receptor. Mapping the local anesthetic binding site. In E. Rubin, K. W. Miller, & S. H. Roth (Eds.), Molecular and cellular mechanisms of alcohol and anesthetics (pp. 588-599). New York: The New York Academy of Sciences.

Levy, R. (1990). Are drugs targeted at Alzheimer's disease useful? Useful for what? British Medical Journal, 300, 1131-1132.

Liston, D., Russo, L., Mena, E. E., & Williams, I. (1988). Tetrahydroaminoacridine is concentrated in brain following intraperitoneal administration. Neuroscience Abstracts, 14, 1224.

London, E. D., Ball, M. J., & Waller, S. B. (1989). Nicotinic binding sites in cerebral cortex and hippocampus in Alzheimer's dementia. Neurochemical Research, 14, 745-750.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, P. J. (1951). Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry, 193, 265-275.

Magleby, K. L., & Pallotta, B. S. (1981). A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. Journal of Physiology, 316, 225-250.

Manalis, R. S. (1977). Voltage-dependent effect of curare at the frog neuromuscular junction. Nature, 267, 366-368.

Marta, M., Castellano, A., Pavone, O. F., Pagella, P. G., Brufani, M., & Pomponi, M. (1988). New analogs of physostigmine: Alternative drugs for Alzheimer's disease? Life Sciences, 43, 1921-1928.

McCallister, R. M., Isaacs, H., Rongey, R., Peer, M., Au, W., Sonkup, S., & Gardner, M. (1977). Establishment of a human medulloblastoma cell line. International Journal of Cancer, 83, 4967-4971.

Mesulam, M. M., Mufson, E. J., & Levey, A. L. (1983). Cholinergic innervation of cortex by the basal forebrain; cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. Journal of Comparative Neurology, 214, 170-197.

Misgeld, U., Muller, W., & Polder, H. R. (1989). Potentiation and suppression by eserine of muscarinic synaptic transmission in the guinea-pig hippocampal slice. Journal of Physiology (London), 409, 191-206.

Mo, N., Dun, N. J., & Karczmar, A. G. (1985). Facilitation and inhibition of nicotinic transmission by eserine in the sympathetic ganglia of the rabbit. Neuropharmacology, 24, 1093-1101.

Neher, E. (1983). The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anæsthetics. Journal of Physiology (London), 339, 663-678.

Neher, E., & Steinbach, J. H. (1978). Local anæsthetics transiently block currents through single acetylcholine-receptor channels. Journal of Physiology (London), 277, 153-176.

Nilsson, L., Adem, A., Hardy, J., Winblad, B., & Nordberg, A. (1987). Do tetrahydroaminoacridine (THA) and physostigmine restore acetylcholine release in Alzheimer brains via nicotinic receptors? Journal of Neural Transmission, 70, 357-368.

Nyback, H., Nyman, H., Ohman, G., Nordgren, I., & Lindstrom, B. (1988). Preliminary experiences and results with THA for the amelioration of symptoms of Alzheimer's disease. In R. Becker & E. Giacobini (Eds.), Current research in Alzheimer therapy New York: Taylor and Francis.

Ojeda, V. J., Mastaglia, F. L., & Kakulas, B. A. (1986). Causes of organic dementia: A necropsy survey of 60 cases. Medical Journal of Australia, 145, 69-71.

Osterrieder, W. (1987). 9-Amino-1,2,3,4-tetrahydroaminoacridine (THA) is a potent blocker of cardiac potassium channels. British Journal of Pharmacology, 92, 521-525.

Papke, R. L., & Oswald, R. E. (1989). Mechanisms of noncompetitive inhibition of acetylcholine-induced single-channel currents. The Journal of General Physiology, 93, 785-811.

Park, T. H., Tachiki, K. H., & Summers, W. K. (1986). Isolation and the fluorometric, high-performance liquid chromatographic determination of tacrine. Analytical Biochemistry, 159, 358-362.

Pascuzzo, G. J., Akaike, A., Maleque, M. A., Shaw, K.-P., Aronstam, R. S., Rickett, D. L., & Albuquerque, E. X. (1984). The nature of the interactions of pyridostigmine with the nicotinic acetylcholine receptor-ionic channel complex. I. Agonist, desensitizing, and binding properites. Molecular Pharmacology, 25, 92-101.

Passagli, A., & Pepeu, G. (1964). Amnestic properties of scopolamine and brain acetylcholine in the rat. International Journal of Neuropharmacology, 4, 291-299.

Penn, R. D., Martin, E. M., Wilson, R. S., Fox, J. H., & Savoy, S. M. (1988). Intraventricular bethanecol infusion for Alzheimer's disease. Results of double-blind and escalating-dose trial. Neurology, 38, 219-226.

Peper, K., Sterz, R., & Bradley, R. J. (1981). Effects of drugs and antibodies on the post-synaptic membrane of the neuromuscular junction. Annals of the New York Academy of Sciences, 377, 519-543.

Perl, D. P., & Pendlebury, W. W. (1987). Neuropathology of Alzheimer's disease and related dementias. In H. Y. Meltzer (Eds.), Psychopharmacology: The third generation of progress (pp. 881-885). New York: Raven Press.

Perry, E. (1988). Acetylcholine and Alzheimer's disease. British Journal of Psychiatry, 152, 737-740.

Perry, E. K. (1986). The cholinergic hypothesis-ten years on. British Medical Bulletin, 42, 63-69.

Perry, E. K. (1987). Cortical neurotransmitter chemistry in Alzheimer's disease. In H. Y. Meltzer (Ed.), Psychopharmacology: The third generation of progress (pp. 887-895). New York: Raven Press.

Perry, E. K., Perry, R. H., Blessed, G., & Tomlinson, B. E. (1977). Necropsy evidence of central cholinergic deficits in senile dementia. Lancet, 1, 189.

Perry, E. K., Smith, C. J., Court, J. A., Bonham, J. R., Rodway, M., & Atack, J. R. (1988a). Interaction of 9-amino-1,2,3,4-tetrahydroamino-acridine (THA) with human cortical nicotinic and muscarinic receptor binding in vitro. Neuroscience Letters, 91, 211-216.

Perry, E. K., Smith, C. J., Xuereb, J. H., Keith, A. B., & Perry, R. H. (1988b). Cholinergic neuropathology and nicotinic receptor binding in the human brain. In F. Clementi, C. Gotti, & E. Sher (Eds.), Nicotinic acetylcholine receptors in the nervous system (pp. 351-365). New York: Springer-Verlag.

Potter, L. T., Ferrendelli, C. A., Hanchett, H. E., Hollifield, M. A., & Lorenzi, M. V. (1989). Tetrahydroaminoacridine and other allosteric antagonists of hippocampal M1 muscarine receptors. Molecular Pharmacology, 35, 652-660.

Read, S. L. (1988). Intra-cerebro-ventricular bethanechol: Dose and response. In R. Becker & E. Giacobini (Eds.), Current research in Alzheimer therapy New York: Taylor and Francis.

Rinne, J. O., Säkö, E., Paljärvi, L., Mölsä, P. K., & Rinne, U. K. (1988). A comparison of brain choline acetyltransferase activity in Alzheimer's disease, multi-infarct dementia, and combined dementia. Journal of Neural Transmission, 73, 121-128.

Rogawski, M. A. (1987). Tetrahydroaminoacridine blocks voltage-dependent ion channels in hippocampal neurons. European Journal of Pharmacology, 142, 169-172.

Ruff, R. L. (1977). A quantitative analysis of local anesthetic alteration of miniature end-plate potential currents and end-plate current fluctuations. Journal of Physiology (London), 264, 89-124.

Sakman, B., & Neher, E. (Eds.). (1983). Single-channel recording. New York: Plenum Press.

Schauf, C. L., & Sattin, A. (1987). Tetrahydroaminoacridine blocks potassium channels and inhibits sodium inactivation in Myxicola. The Journal of Pharmacology and Experimental Therapeutics, 243, 609-613.

Schubert, D., Harris, A. J., Devine, C. E., & Heinemann, S. F. (1974). Characterization of a unique muscle cell line. Journal of Cell Biology, 61, 398-413.

Scuka, M. (1987). The effect of anticholinesterase drugs on the ionophoretically-evoked end-plate currents. Pflügers Archiv, 410, 428-432.

Seifert, S. A., & Eldefrawi, M. E. (1974). Affinity of myasthenia drugs to acetylcholinesterase and acetylcholine receptor. Biochemical Medicine, 10, 258-265.

Sharpless, N. S., & Thal, L. J. (1985). Plasma physostigmine concentrations after oral administration. Lancet, 1, 1397-1398.

Shaw, K.-P., Aracava, Y., Akaike, A., Daly, J. W., Rickett, D. L., & Albuquerque, E.X. (1985). The reversible cholinesterase inhibitor physostigmine has channel-blocking and agonist effects on the acetylcholine receptor-ion channel complex. Molecular Pharmacology, 28, 527-538.

Sherby, S. M., Eldefrawi, A. T., Albuquerque, E. X., & Eldefrawi, M. E. (1985). Comparison of the actions of carbamate anticholinesterases on the nicotinic acetylcholine receptor. Molecular Pharmacology, 27, 343-348.

Shutske, G. M., Pierrat, F. A., Cornfeldt, M. L., Szewczak, M. R., Huger, F. P., Bores, G. M., Haroutunian, V., & Davis, K. L. (1988). (±)-9-Amino-1,2,3,4-tetrahydroacridin-1-ol. Potential Alzheimer's disease therapeutic of low toxicity. Journal of Medical Chemistry, 31, 1278-1279.

Sine, S. M., & Steinbach, J. H. (1988). Function of mammalian nicotinic acetylcholine receptors: Agonist concentration dependence of single channel current kinetics. In J.F. Hoffman & G. Giebisch (Eds.), Current topics in membranes and transport (pp. 133-145). New York: Harcourt Brace Jovanovich.

Sterz, R., Peper, K., Simon, J., Ebert, J. P., Edge, M., & Bradley, R. J. (1986). Agonist and blocking effects of choline at the neuromuscular junction. Brain Research, 385, 99-114.

Stevens, D. R., & Cotman, C. W. (1987). Excitatory actions of tetrahydro-9-aminoacridine (THA) on hippocampal pyramidal neurones. Neuroscience Letters, 79, 301-305.

Strecker, G. J., & Jackson, M. B. (1989). Curare binding and the curare-induced subconductance state of the acetylcholine receptor channel. Biophysical Journal, 56, 795-806.

Summers, W. K. (1989). Tacrine in the treatment of Alzheimer's disease. A clinical update and recent pharmacologic studies. European Neurology, 29 (Supplement 3), 28-32.

Summers, W. K., Majorski, L. V., Marsh, G. M., Tachiki, K., & Kling, A. (1986). Oral tetrahydrohydroaminoacridine in long term treatment of senile dementia, Alzheimer type. New England Journal of Medicine, 315, 1241-1245.

Summers, W. K., Viesselman, J. O., Marsh, G. M., & Candelora, K. (1981). Use of THA in treatment of Alzheimer-like dementia: Pilot study in twelve patients. Biological Psychiatry, 16, 145-153.

Taylor, P. (1985). Neuromuscular blocking agents. In A. G. Gilman, L. S. Goodman, T. W. Rall, & F. Murad (Eds.), The pharmacological basis of therapeutics (pp. 222-235). New York: Macmillan Publishing Company.

U.S. Food and Drug Administration (1992). Treatment protocol approved for THA. FDA medical bulletin, 22, 9-10.

Wachtel, R. E. (1988). Ketamine decreases the open time of single-channel currents activated by acetylcholine. Anesthesiology, 68, 563-570.

Wachtel, R. E. (1990). Comparison of anticholinesterases and their effects on acetylcholine-activated ion channel. Anesthesiology, 72, 496-503.

Watson, C. P. (1979). Clinical similarity of Alzheimer and Creutzfeldt-Jakob disease. Annals of Neurology, 6, 368-369.

Wecker, L., Dettbarn, W., & Schmidt, D. E. (1978). Choline administration: Modification of the central actions of atropine. Science, 199, 86-87.

White, P., Goodhardt, M. J., Keet, J. P., Hiley, C. R., Carasco, J. H., Williams, I. E. I., & Bowen, D. M. (1977). Neocortical cholinergic neurons in elderly people. Lancet, 1, 668-671.

Whitehouse, P. J., Martino, A. M., Marcus, K. A., Zweig, R. M., Singer, H. S., Price, D. L., & Kellar, K. J. (1988). Reduction acetylcholine and nicotine binding in several degenerative diseases. Archives of Neurology, 45, 722-724.

Whitehouse, P. J., Parhad, I. M., Hedrenn, J. C., Clark, A. W., White, C. L., Struble, R. G., & Price, D. L. (1983). Integrity of the nucleus basalis in normal aging. Neurology, 33, 159.

Whitehouse, P. J., Price, D. L., Clark, A. W., Coyle, J. T., & DeLong, M. K. (1981). Alzheimer disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis. Annals of Neurology, 10, 122-126.

Whitehouse, P. J., Price, D. L., Clark, A. W., Coyle, J. T., & DeLong, M. K. (1982). Alzheimer's disease and senile dementia-Loss of neurons in the basal forebrain. Science, 215, 1237-1239.

Wisniewski, H. M., Rabe, A., Zigman, W., & Silverman, W. (1989). Neuropathological diagnosis of Alzheimer disease. Journal of Neuropathology and Experimental Neurology, 48, 606-609.

Wurtman, R. J., Hefti, F., & Melamed, E. (1981). Precursor control of neurotransmitter synthesis. Pharmacological Reviews, 32, 315-335.

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