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Physostigmine Block of Ion Channels Activated by Acetylcholine in BC3H1 Cells

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SUMMARY

Single-channel recording techniques have been used to study the effects of physostigmine on the kinetics of ion channels activated by acetylcholine in BC3H1 mouse tumor cells grown in culture. Physostigmine reduced mean channel open time, with 50% reduction occurring at about 7 μM physostigmine. Although openings did not appear to occur in bursts, channel closed-time distributions exhibited a new component 7–8 msec in duration. The area of this component, but not its time constant, increased with higher concentrations of physostigmine. Results are consistent with a simple sequential channel-blocking model in which the new closed-time component represents a blocked state of the channel. Membrane hyperpolarization increased the potency of physostigmine in reducing channel open time and also prolonged the duration of the blocked state. The voltage dependence of physostigmine block was unexpected, because physostigmine is uncharged at physiological pH.

Numerous cholinesterase inhibitors, including edrophonium, pyridostigmine, neostigmine, physostigmine, and methanesulfonyl fluoride (1–4), interact directly with the open state of ion channels activated by ACh. These effects are independent of inhibition of cholinesterase.

Physostigmine, a carbamate cholinesterase inhibitor that is uncharged at physiological pH and readily crosses the blood-brain barrier, appears to act by blocking the open channel. At the frog neuromuscular junction, physostigmine causes the decay of endplate currents to become biphasic and it induces flickering of ACh-activated single-channel currents, resulting in an apparent reduction in single-channel conductance (2). Physostigmine also inhibits binding of labeled histrionicotoxin to the agonist-activated conformation of ACh channels in *Torpedo* membranes (1). These effects are all consistent with open channel block.

However, the actions of several compounds assumed to be channel blockers have been found to conflict with a simple channel-blocking model, when examined in detail. The effects of both local anesthetics (5, 6) and general anesthetics (7)[1] on ACh-activated channels cannot be completely explained by a sequential channel-blocking model but require cyclic models in which blocked channels can close directly, without passing back through the open state.

The purpose of these experiments was to determine whether the effects of physostigmine on ACh-activated channels are indeed consistent with a simple channel-blocking mechanism or whether a more complex model is necessary to explain drug interactions with the channel.

Materials and Methods

The methods used in these experiments have been reported previously (8, 9). Patch-clamp techniques were used to record single-channel currents activated by ACh from cell-attached patches of BC3H1 mouse tumor cells grown in culture. Differentiated cells plated onto coverslips were bathed in a solution containing 100 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. Electrodes were pulled in two stages, on a Narishige PP-88 puller, from WPI TW150 borosilicate glass capillaries. Electrodes had resistances of 2–5 MΩ and were filled with the bathing solution plus 200–250 nM ACh and 2–50 μM physostigmine salicylate (Antilirium). Experiments were performed at controlled room temperature (20–23°).

For most experiments, channels were measured with the patch hyperpolarized +75 mV relative to cell resting potential, which was estimated to be −60 mV based on a reversal potential of 0 mV for ACh-activated currents. Channels were recorded with a List EPC-7 patch-clamp amplifier and stored on a Sony Betamax recorder with a Sony PCM-1 digital audio processor. Signals were later played back through a Frequency Devices 902LPF1 low-pass Bessel filter (set at 5 kHz), digitized at 20 μsec/point, and stored on an AST Premium 286 computer equipped with a LabMaster analog-to-digital conversion board. Single-channel events were analyzed using the pCLAMP software package (Axon Instruments) to determine channel amplitude, open duration, and closed duration (time between events). Channel open-

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[1] Wachtel, R. E. Volatile anesthetics alter the kinetics of nicotinic acetylcholine channels in BC3H1 cells. Submitted for publication.

ABBREVIATIONS: ACh, acetylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

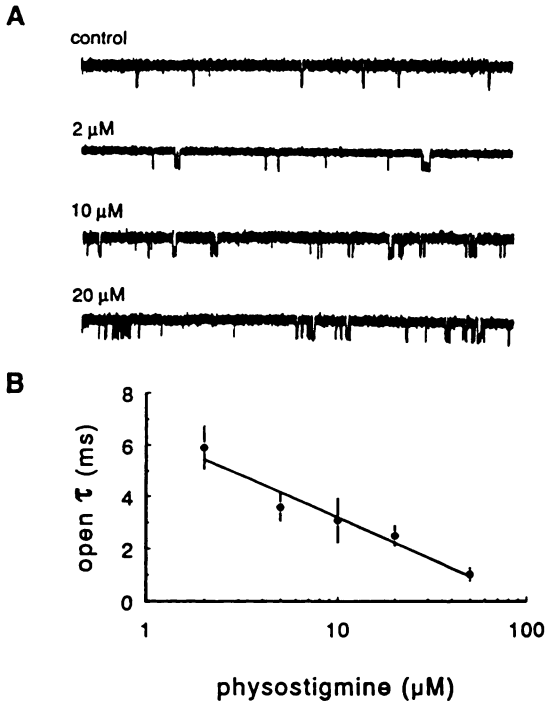


Fig. 1. A, Representative recordings of single-channel events. Each *trace* is 2 sec in duration. Channels were recorded with the patch pipet hyperpolarized +75 mV relative to cell resting potential. B, Physostigmine decreased channel open time. In the absence of drug, τ = 7.34 ± 1.57 msec (n = 30 patches). Open time was reduced 50% at about 7 μM physostigmine. Each *point* is the mean ± standard deviation of five to seven patches. The *line* is a least squares fit.

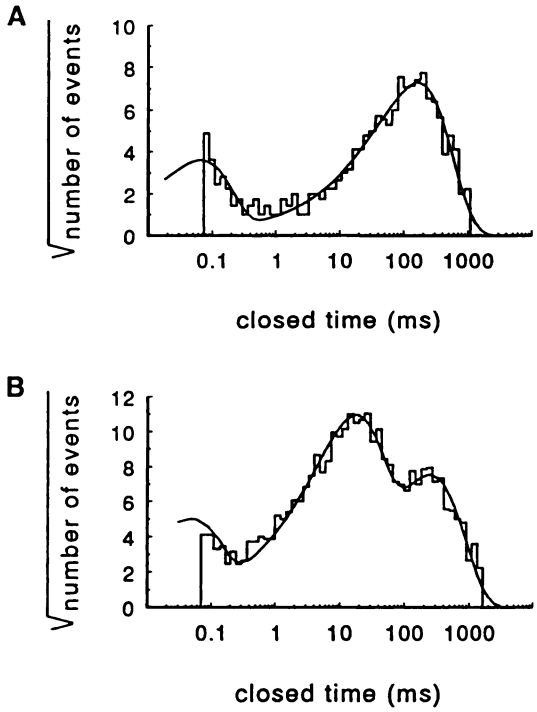


Fig. 2. Representative closed-time histograms. Probability density functions are superimposed on the data. A, Distribution of closed times in control patch is well described by two exponential components, with τ_f = 0.07 msec, τ_s = 166 msec, and A_f = 0.18 (n = 760 events). B, Patch exposed to 20 μM physostigmine. Note prominent intermediate component; τ_f = 0.05 msec, τ_i = 16.8 msec, τ_s = 245 msec, A_f = 0.13, and A_i = 0.58 (n = 2312 events). Differences in τ_s probably arise from variations in the number of active channels in each patch.

time distributions were described as sums of one or two exponential components with time constants τ_f and τ_s ($\tau_f < \tau_s$) and fast component area A_f, estimated by the method of maximum likelihood (10, 11). Channel closed-time distributions were described as sums of two (control) or three (physostigmine) exponential components with time constants τ_f, τ_i, and τ_s ($\tau_f < \tau_i < \tau_s$) and relative areas A_f, A_i, and A_s ($A_f + A_i + A_s = 1$), estimated by the method of maximum likelihood. The number of exponential components was determined using the Akaike Information Criterion (12). Because the recording system was unable to detect events less than about 0.08 msec in duration, open-time and closed-time distributions were corrected for missed events less than 0.1 msec in duration. Patches containing frequent multiple openings or with τ_s (closed time) of <100 msec were excluded from analysis. Whenever possible, at least 1000 events were analyzed for each patch. All recordings were assumed to be stationary, because experiments in which data were analyzed in 2-min segments showed no changes in time constants or areas over a 10-min period.

Although patches contained more than one active channel, all openings within a burst were assumed to arise from activation of a single channel. Two methods were used define a burst and calculate open time/burst. 1) A burst was defined as a group of openings separated by either fast or intermediate closed times. Open time/burst was then calculated from the number of openings/burst [equal to $1 + ((A_f + A_i)/A_s) = 1/A_s$] times the average duration of each opening. 2) A burst was also defined as a group of openings separated by closed times shorter than a critical time t_c. The value of t_c for each patch was determined by solving the equation $\exp(-t_c/\tau_i) + \exp(-t_c/\tau_s) = 1$ and ranged from 15 to 40 msec in the presence of physostigmine. The fraction of intermediate closed times (within a burst) that are misclassified as long closed times (between bursts) is thus equal to the fraction of long closed times misclassified as intermediate (13). Open time/burst was then measured directly by summing the duration of individual openings within each burst.

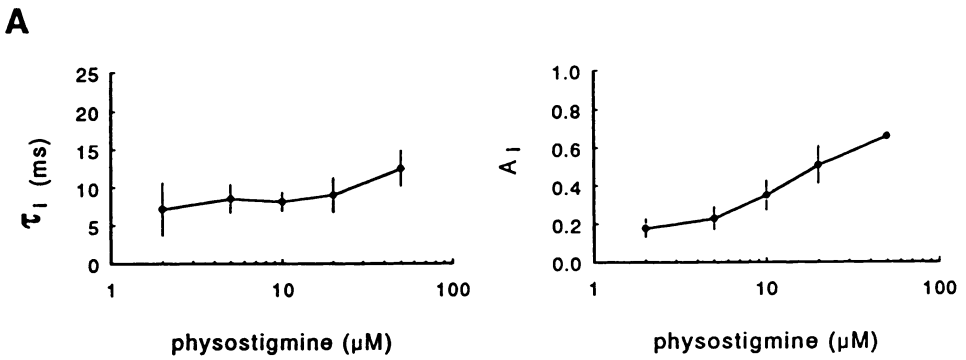
Results

Mean current amplitude was not altered by physostigmine. Single-channel current amplitude at 20 μM physostigmine was 2.48 ± 0.17 pA (mean ± standard deviation from n = 8 patches), compared with 2.59 ± 0.24 pA (n = 35) in controls.

The effects of increasing concentrations of physostigmine are shown in Fig. 1. In the compressed time scale of Fig. 1A, individual opening events appear grouped together in bursts.

The duration of individual opening events was reduced by physostigmine in a concentration-dependent manner (Fig. 1B). Channel open-time distributions were usually described as sums of two exponential components, although a faster component could not be resolved at higher physostigmine concentrations. In controls, the faster component had a τ of only 0.18 ± 0.07 msec and an area of 0.40 ± 0.10 (n = 28). Because this faster component contributed only a small fraction of the total charge transferred during channel opening, it was not considered further. A single time constant τ is therefore used to describe channel open-time distributions, signifying the time constant of single-exponential distributions or the slower time constant of double-exponential distributions. In the absence of drug τ was 7.34 ± 1.57 msec (n = 30), and τ was reduced 50% by approximately 7 μM physostigmine.

Representative closed-time histograms obtained in the absence and presence of physostigmine are shown in Fig. 2. Control patches were usually described as sums of two exponential components, although a very small intermediate component was observed in about half of control patches, with τ_i of 1.97 ± 1.35 msec and A_i of 0.08 ± 0.03 (n = 14). With 20 μM



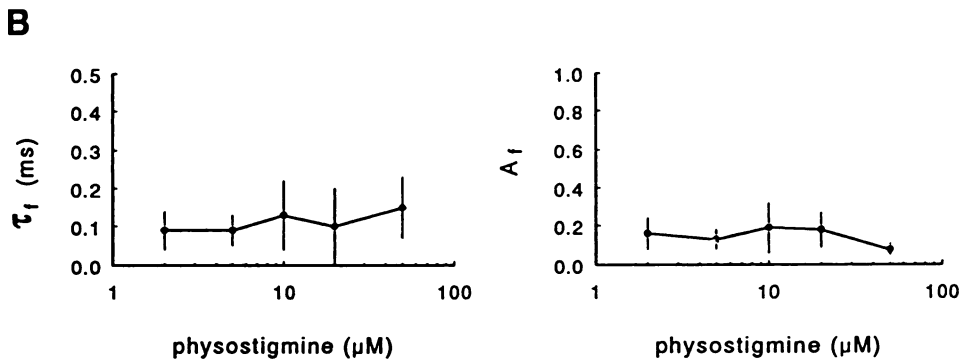


Fig. 3. Effects of increasing concentrations of physostigmine on the intermediate and fast components of channel closed-time distributions. A, The τ_i value was not consistently affected by physostigmine but was increased slightly at the highest concentration; A_i increased as the concentration of physostigmine was raised and the intermediate component became the dominant peak in closed-time distributions. Although a small intermediate component was sometimes observed in control patches, its time constant did not correspond to that of the intermediate component observed with physostigmine. B, Neither τ_f nor A_f was affected by physostigmine. In control, τ_f = 0.19 ± 0.17 msec and A_f = 0.23 ± 0.14 (n = 28 patches). Each *point* is the mean ± standard deviation of five to seven patches.

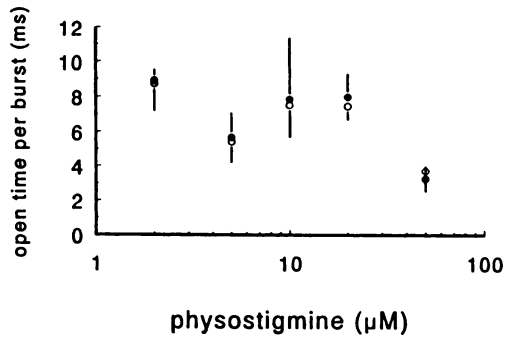


Fig. 4. Total open time/burst was not affected by physostigmine. ●, Open time/burst was calculated from the product of number of openings/burst, equal to $1/A_a$, multiplied by average open time. In control this value was 9.57 ± 2.60 msec (n = 26). ○, Open time/burst was calculated by summing the duration of individual opening events. In control this value was 8.82 ± 2.17 msec (n = 26). This method of calculating open time/burst yields slightly smaller values because it is more sensitive to missed events.

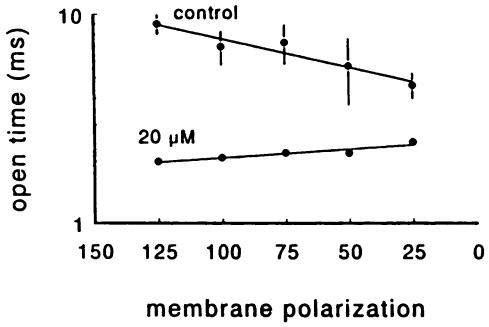


Fig. 5. Voltage dependence of reduction in channel open time produced by physostigmine. Membrane polarization refers to pipet potential and thus corresponds to hyperpolarization beyond cell resting potential. Physostigmine was relatively more effective at reducing channel open time as the membrane was hyperpolarized. Each control value is the mean ± standard deviation of four to six patches. Data for 20 μM physostigmine are from a representative cell.

physostigmine in the patch pipet, however, an intermediate component was quite prominent. This component overwhelmed the small intermediate component that might be present in controls but did not have the same time constant. It appeared to represent a new closed state of the channel.

The effects of increasing concentrations of physostigmine on the new intermediate component of closed-time distributions are shown in Fig. 3A. The time constant of this component averaged about 7–8 msec and was independent of physostigmine concentration, although a small increase was seen at 50 μM, the highest concentration that could be studied. In contrast, A_i was highly dependent on drug concentration, increasing from 0.18 ± 0.05 at 2 μM to 0.66 ± 0.02 at 50 μM physostigmine.

The effects of physostigmine on the fast component of closed-time distributions are shown in Fig. 3B. Physostigmine had no consistent effect on either τ_f or A_f. The effects of physostigmine on the slow time constant of closed-time distributions were not analyzed in detail. The slow component does not necessarily reflect a rate of transition between states of the channel but may exhibit variability between patches due to differences in the number of active channels present.

In summary, physostigmine induced a new component in channel closed-time distributions. The area of this component, but not its time constant, increased with drug concentration. Results can be interpreted in terms of a sequential channel-blocking model (14–16):

$$\text{closed} \rightleftharpoons \text{closed} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{open} \underset{b}{\overset{fc}{\rightleftharpoons}} \text{blocked}$$

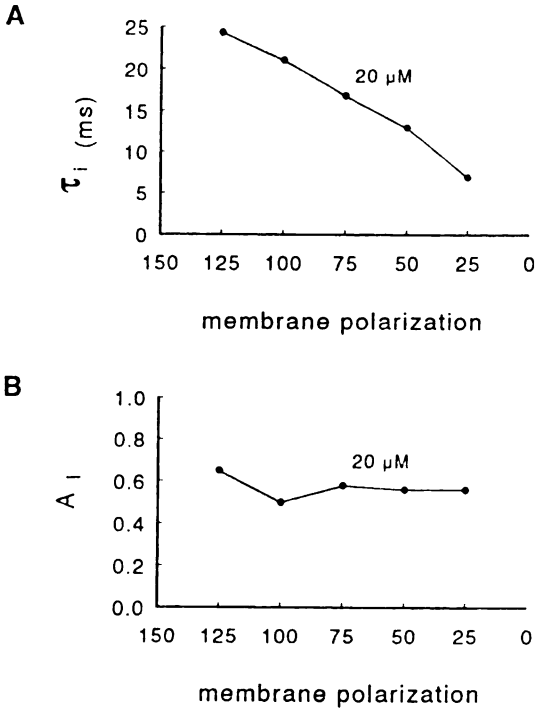


Fig. 6. Voltage dependence of physostigmine effects on the intermediate component of closed-time distributions. Membrane polarization refers to pipet potential and thus corresponds to hyperpolarization beyond cell resting potential. Data are from a representative cell exposed to 20 μM physostigmine. A, The τ_i value, or the duration of the blocked state, increased at hyperpolarized potentials. B, A_i was not affected by voltage. Similar results were obtained at other drug concentrations.

where c is blocker concentration, and α, β, f, and b are rate constants.

The intermediate closed times observed with physostigmine may represent a blocked state of the channel. Channel openings are shortened because the channel can either close normally or become blocked. The rate constant for the blocking reaction is $f = 1.6 \times 10^7$ M^{-1} sec^{-1}, determined from the slope of a plot of open $time^{-1}$ as a function of drug concentration. Although a channel blocker typically causes openings to appear grouped together in bursts as the channel chatters back and forth between open and blocked states, openings observed in the presence of physostigmine do not obviously appear to occur in bursts, because of the relatively long duration of the blocked state.

The sequential model predicts that total open time/burst should be unchanged in the presence of a blocking drug (5, 17). Fig. 4 shows that open time/burst was not substantially decreased by physostigmine, except at the highest concentration. Although there was a great deal of scatter in the data points due to the large number of calculations involved in obtaining these numbers, the results are in marked contrast to other studies of putative blocking agents that show dramatic reductions in open time/burst. These data obtained with physostigmine are unusual, in that they are consistent with a sequential blocking model, whereas many other drugs deviate from the model by reducing open time/burst.

Another unusual feature of channel block by physostigmine is its voltage dependence. Fig. 5 shows that increasing the potential across the membrane patch enhanced the physostigmine-induced reduction in channel open time. Channel open time was normally longer at hyperpolarized potentials, due to an inherent voltage dependence of the channel-closing reaction, but this trend was reversed at 20 μM physostigmine.

The effect of membrane voltage on the intermediate component of channel closed-time distributions is shown in Fig. 6. The τ_i value, which was independent of physostigmine concentration, increased as the membrane patch was hyperpolarized. Thus, time spent in the blocked state was enhanced by hyperpolarization. In contrast, A_i, which increased with increasing drug concentration, was not affected by voltage. Thus, the number of transitions to the blocked state was not altered by hyperpolarization.

Discussion

Physostigmine produces a new component in channel closed-time distributions. The relative area of this component increases with drug concentration, but its time constant is independent of drug concentration. In addition, total open time/burst is not reduced by physostigmine except at a very high concentration. These results are completely consistent with a sequential blocking model in which molecules of drug occlude the open channel. The constancy of open time/burst means that a sequential model is appropriate; after dissociation of drug, the channel passes back through the open state before closing.

These findings are rather unusual, however, because a number of other agents originally thought to be channel blockers were subsequently shown to decrease open time/burst. A sequential blocking model is unable to explain the kinetics of numerous agents that cause channel openings to appear grouped together in bursts, such as local anesthetics (6) and general anesthetics (7),[1] and cyclic models must be invoked to explain decreases in open time/burst.

Another unexpected result of these studies is the voltage dependence of channel block by physostigmine. Physostigmine was more effective at reducing channel open time as the potential across the membrane was increased, consistent with earlier findings on endplate currents by Shaw *et al.* (2). In addition, the duration of the blocked state, as determined by the time constant of the intermediate component, also increased with hyperpolarization. Although the potency of numerous drugs, including *d*-tubocurarine (18), quinacrine (19), and local anesthetics (15, 20), in reducing channel open time is also enhanced by membrane hyperpolarization, these substances tend to be positively charged. The increase in channel block with hyperpolarization is thought to arise from passive movement of positive charges down their electrical gradient toward the inside of the membrane (18). This explanation cannot account for the voltage dependence of physostigmine block, however, because the compound has a pK_a of 6.1 (21) and is essentially uncharged at the pH of these experiments.

An alternative explanation for the voltage dependence of physostigmine block is an alteration in the state of ionization of a membrane protein associated with the site of action of the drug. A similar explanation has been proposed to explain the identical voltage dependence of procaine block of endplate channels when drug is applied to either the extracellular or cytoplasmic surface (22). These results also lead to speculation that the uncharged physostigmine may reach its site of action via the lipid phase of the membrane, rather than the lumen of the open channel. Even though results are consistent with a

channel-blocking model, this model may not be an accurate description of binding at the molecular level.

Acknowledgments

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