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The Nature of the Interactions of Pyridostigmine with the Nicotinic Acetylcholine Receptor-Ionic Channel Complex

II. Patch Clamp Studies

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SUMMARY

Patch clamping of myoballs to record single channels was performed to examine the interaction of the anticholinesterase agent pyridostigmine (Pyr) with the acetylcholine (ACh) receptor-ion channel complex. Single ACh channel currents were recorded from tissue-cultured muscle cells of neonatal rats (myoballs). Pyr (50–100 μM) decreased the frequency of channel-opening events activated by ACh, and induced a modified form of the ACh channel currents. Channel conductance was lower in the presence of Pyr, and channel lifetime remained unaltered or only slightly prolonged. In addition, channel openings were frequently interrupted by fast flickers in the presence of Pyr. Higher concentrations (200 μM–1 mM) of the drug induced irregular waves of bursting activity during the initial phase of the application, and, subsequently, significantly reduced the frequency of channel openings. Infrequent channel openings with low conductance were observed in the patch when the micropipette was filled with Pyr alone. These results suggest that, in addition to its anticholinesterase activity, Pyr reacts with the ACh receptor, and both alone or in combination with ACh induces an altered, desensitized species of the nicotinic receptor-ion channel complex.

INTRODUCTION

The reaction of ACh[3] with the nicotinic receptor initiates a series of conformational changes resulting in activation of the ionic channel which is an integral component of the receptor macromolecule (1). Such an effect of ACh initiates a current flowing through single ionic channels which can be measured by using the extracellular patch clamp technique recently developed by Neher and Sakmann (2). Refinement of this technique has allowed a much better signal-to-noise ratio at bandwidths of 1–3 KHz by forming a tighter seal between the micropipettes and the ACh receptor-rich membrane. The resistance of the seal between the specially prepared micropipette and the cell surface ranged from 1 to 20 gigaohm (3–5).

The studies described in the companion paper (6) using voltage clamp, fluctuation analysis, and binding techniques demonstrated that Pyr, an agent which reversibly blocks AChE, reduced the peak amplitude and prolonged the time course of the miniature end-plate currents, lengthened the mean channel lifetime, and markedly reduced single-channel conductance of the nicotinic receptor ionic channel complex as revealed by fluctuation analysis. In addition, the agent increased the affinity of ACh to its binding site and generated desensitized conformations, coupled with a weak agonistic activity. These actions of Pyr on the nicotinic ACh receptor ionic channel complex were not caused by AChE inhibition. In light of these findings we decided to investigate further the action of Pyr on the single channels of the nicotinic receptor using a patch clamp technique. For these studies we used cultured "myoballs" (7) derived from neonatal rat hind limb muscles.

METHODS

Patch clamp technique. All experiments were performed at 10–11° on myoballs cultured from hind limb muscles of 1- to 2-day-old rat

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[3] The abbreviations used are: ACh, acetylcholine; Pyr, pyridostigmine; AChE, acetylcholinesterase; TTX, tetrodotoxin; α-BGT, α-bungarotoxin; BSS, balanced salt solution.

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pups [DUB (SD), Dominion Laboratories)]. The cell culturing procedure was adapted from that described by Giller *et al.* (8) for mouse tissues. The cells, seeded on Thermanox plastic cover slips coated with acid-soluble collagen (Calbiochem), were supplied initially with a nutrient medium containing 10% fetal calf serum, 10% heat-inactivated horse serum, and 80% modified Eagle medium (GIBCO, Lot 320-1935). After 4–5 days, the fetal calf serum was omitted from the nutrient medium, and proliferation of fibroblasts was arrested by the addition of 5-fluoro-2′-deoxyuridine (15 μg/ml) and uridine (35 μg/ml) for 1 day. The cultures were incubated at 34° in a water-saturated atmosphere of 10% CO_2/90% air, and the medium was changed twice weekly. The myoballs used in this study formed spontaneously (i.e., without addition of colchicine) in cultures which were incubated for 1–2 weeks. On removal of cultures from the incubator, the nutrient medium was replaced with Hanks' BSS (millimolar composition: NaCl 137, KCl 5.4, $NaHCO_3$ 4.2, $CaCl_2$ 1.3, $MgSO_4$ 0.81, KH_2PO_4 0.44, Na_2HPO_4 0.34, D-glucose 5.5) to which was added 0.001% phenol red, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), and sucrose to adjust the osmolarity to 340 mosmolar. This bathing solution also contained 3×10^{-7} M TTX in order to abolish the contraction of myoballs.

The patch pipettes were pulled on a vertical electrode puller (David Kopf) using microhematocrit capillary tubes of 75 mm length and 1.1–1.2 mm inner diameter. The micropipette was prepared in two stages according to the procedure described by Hamill *et al.* (4). In the first step, the capillary tube was thinned with a pulling length of 9 mm. The second step fractured the narrow portion of the capillary with a tip diameter <2 μm. The pipette shanks were coated with Sylgard and the pipette tips fire-polished by the heat emitted from the glass-covered tip of a V-shaped platinum filament (about 75 μm in diameter). The filament was connected to a large brass heat sink such that only the glass-covered portion of the filament reached high temperature. The microelectrodes used in the experiments had an inner tip diameter of less than 1 μm, and resistances ranged from 2 to 6 Mohm. The patch microelectrodes were filled with Hanks' BSS containing 3×10^{-7} M TTX and 3×10^{-8} to 2×10^{-7} M ACh. In other experiments, ACh was replaced with 5×10^{-5} to 10^{-3} M Pyr or the combination of both agents at suitable concentrations.

The tip of the patch electrode was pressed against a cell membrane under microscopic (×400 Hoffman modulation optics) observation. Gigaohm seals were achieved by applying gentle suction through the patch electrode. After establishment of 5–15 gigaohm seals, the potential inside the pipette (i.e., extracellular space of the patched membrane) was adjusted to the desired holding potential. Experiments were carried out with the cell-attached patch ("on the myoball") or the cell-free inside-out patches. In the latter case, the electrode was removed from the myoball along with a tear-off patch of cell membrane, which was then exposed to the air for a brief time. This procedure provided a cell-free patch with the cytoplasmic face of the membrane exposed to the bath solution. Single-channel currents were measured with a LM-EPC-5 patch-clamp system (List-Electronics, West Germany). The single-channel currents were filtered to 1–3 KHz (second-order, Bessel low pass) and then monitored on a digital oscilloscope and Mingograf recorder. These records were simultaneously stored on FM magnetic tape (Racal, 15 ips, dc–5 KHz) for computer analysis.

Computer analysis. Automated analysis of patch clamp data was performed on a PDP 11/40 (Digital Equipment Corporation, Maynard, Mass.) equipped with 28 K words of core memory. All programs were written in FORTRAN IV or MACRO-11 assembly language and run in an RT-11 operating system environment. Portions of the program were based on the work of Sachs *et al.* (9).

Data were sent to the computer from FM tape and digitized at 2 KHz by an LPS-11 (Digital Equipment Corporation) 12-bit analogue to digital converter. The data were sent through a fourth-order Butterworth (low-pass) filter (1–3 KHz) to eliminate high-frequency noise and improve the signal-to-noise ratio. Files of 16,384 contiguous points (8.192 sec) were digitized and stored on RL02 (10 megabytes) or RK05 (2.5 megabytes) hard discs, or standard magnetic tape for later analysis. The data were edited prior to analysis, and records containing large dc shifts or oscillations in the baseline were discarded.

Identification of single-channel currents was accomplished using a program, RHONDA, coded in FORTRAN IV, with assembly language subroutines for time-dependent portions of the analysis. Each file was divided into records of 2048 points, and the baseline was determined by finding the first local maximum in the number of zero crossings. This was accomplished by determining the minimum point (all data were sent such that channel openings were of positive polarity) in the record and progressively incrementing a zero crossing detector until the first local maximum was found. Although this method worked reliably, it occasionally failed with records containing extremely long channels or high frequencies of openings. The standard deviation of the baseline was determined for the first record in each file, using a "bootstrap" technique, as follows: (a) baseline standard deviation was first determined using all points in the record, (b) channel openings were then eliminated from the baseline by determining the maximal point in the record and removing all points contiguous to this point and greater than the baseline, and (c) the standard deviation of the record was then redetermined. This process was repeated until the difference in the standard deviation between subsequent determinations was less than a given value. Each record in a file was sequentially analyzed for channel openings. A channel was considered open when a data point exceeded a set number of standard deviations from the baseline. Subsequent points in the record were then scanned until the signal returned to within a given number of standard deviations from the baseline. The number of standard deviations was chosen to represent about 50% of the unitary conductance. This was considered a channel closure. It is important to note that a "flicker" within an open channel, i.e., a short-duration transition from the open to closed state and back, terminated the open channel event if the flicker reached the closing threshold. Thus, a long channel could be broken up into several adjacent shorter channels by flickers. The maximal point within the interval between an opening and closing was then determined. If this value exceeded a given number of standard deviations above the current amplitude (as would be the case for a multiple-channel opening), the lifetime data for this event (the time between opening and closing) were discarded. Otherwise, the lifetime data were stored in an array for later analysis. If more than 10% of the channels analyzed from any particular cell were multiple openings, the data were used for estimates of channel amplitude. The amplitude of the event was then determined by either finding a local maximum in the zero crossing from the maximal point (incrementing, the short-duration search in the negative direction) or by averaging the points during the open interval. The choice of methods for determining amplitude was dependent on lifetime, the former method used for channel lifetimes greater than 20 sampling intervals. The amplitude was then used to update the current amplitude estimate. A second parameter generated at this phase was a total amplitude histogram. The difference between each point in the file and baseline was converted to a current value (picoamps) and binned in fixed 0.05-pamp bins.

Throughout this phase of analysis the performance of the program was monitored with both visual and audio indicators. Each record of 2048 points was displayed on a Tektronix 603 storage oscilloscope and cursors placed on channel boundaries. If a channel opening was considered a single-channel event by the program, a tone was emitted indicating that the open time for the event had been stored. Alterations of these parameters were facilitated by storing the parameters in a disc file, thus allowing easy user modification during the interactive portion of the program. Once the parameters were optimized, a large number of files could be analyzed in batch mode with minimal user interactions.

Channel lifetime histograms were made by sorting and binning the lifetime data determined by the program RHONDA. The channel lifetimes were usually sorted into 50 bins, the first bin starting at 500 μsec, and the bin increment being either 2.5 or 5 msec, depending on the mean channel lifetime. The histograms were always displayed

normalized such that each bin amplitude was a percentage of the largest bin. The logarithm of the bin amplitudes was then calculated and the regression line determined for a given number of bins, which usually included more than 90% of the channel lifetimes. Average channel lifetime was determined by two methods. From the regression line, one estimate of average lifetime could be determined by taking the reciprocal of the slope. This method assumed that the lifetime distribution was adequately fit by a single exponential function. The arithmetic mean of the channel lifetimes was also taken for comparison. Generally, there was a very good agreement between these two determinations. The value from the regression analysis was used in this study.

Drugs. ACh chloride (Sigma Company) solutions were prepared from the crystalline chloride salt for every experiment. Pyr bromide (Hoffmann-La Roche) and TTX (Sigma Chemical Company) were diluted daily from stock solutions of 10^{-2} M and 3×10^{-4} M, respectively. All drug solutions were passed through a Millipore filter (0.2 μm) prior to addition to bath solutions or to the micropipette.

Statistics. The statistical analysis was similar to that described in the preceding paper (6).

RESULTS

Conditions of application of Pyr to the nicotinic receptor-ion channel complex. Pyr was studied at various concentrations on the rat myoball under different conditions of patch clamping and drug application. In each case, the drug was dissolved in Hanks' BSS with or without ACh. Initially the drug was contained in the patch micropipette and ACh channel currents were recorded subsequent to microelectrode establishing a gigaohm seal on the intact cell. In other experiments, a cell-free patch was used, again with drug in the micropipette. The cell-free patch offered the advantage that the transmembrane potential could be determined directly. We also superfused Pyr in the bath using both cell-attached and inside-out preparations.

Alteration of the ACh channels produced by Pyr in the micropipette. Single-channel currents with rectangular shape were observed after the establishment of a gigaohm seal between the microelectrode containing ACh and the surface of the myoball. The baseline noise level was 0.2–0.5 pamp (peak to peak with 1-KHz low-pass filter) when electrode shanks were coated with Sylgard. In inside-out preparations, ACh primarily produced channel openings with an amplitude of 2.0 ± 0.03 pamp (mean ± standard error, $n = 4$ myoballs) at the holding potential of −100 mV. The mean channel lifetime with this amplitude was 27.5 ± 2.9 msec ($n = 4$). Larger (3.1 pamp) and smaller amplitude channels (1.0 pamp) were also recorded, but their frequencies were insufficient to allow an estimation of their significance (5). In addition, in a few rare cases even recordings of single ACh (100 nM) channels from cell-attached patches (on the myoball) also disclosed a variety of channel amplitude. Single-channel currents recorded in these preparations had channel amplitudes (intermediate size) ranging from 1.3 pamp to 2.4 pamp (mean = 1.9 ± 0.3 pamp, $n = 5$) at the holding potential of +60 mV (pipette interior). Mean channel lifetime in this condition was 26.0 ± 4.8 msec ($n = 5$). Although other channels with larger or smaller amplitudes were sometimes evident, analysis was restricted to channels of this intermediate conductance. The amplitude of the channel currents either from cell-free "inside-out" or cell-attached patches was voltage-dependent (see Figs. 1 and 7).

An interesting finding observed in myoballs was an increase in channel opening frequency with membrane hyperpolarization; i.e., as the membrane potential became more negative, a significant increase in channel opening frequency occurred (Fig. 1). Indeed, in seven control cells examined, the frequency of channel opening events showed an apparent dependence on membrane potential (Table 1). Similar observations have also been made by others (10, 11). There are many possibilities which could explain this phenomenon, among others, an increase in the forward rate constant for channel opening (β or k_2) with hyperpolarization. Another but more remote possibility is that the rate constant of agonist binding is voltage-dependent. The detailed analysis of this phenomenon is not within the scope of this paper, but is now the subject of further analysis.[4]

Figure 2 shows the alteration of ACh-induced channels by Pyr. Pyr (50 μM) in combination with ACh (100 nM) induced the appearance of channels with marked flickering but with lifetime unaltered when compared with that produced by ACh alone. Indeed, over 80% of the channel openings were interrupted frequently by short gaps (flickering). Under this experimental condition, the number of channels with flickering increased as a function of time of exposure (between 2 and 6 min after the

[4] A. Akaike, Y. Aracava, and E. X. Albuquerque, unpublished results.

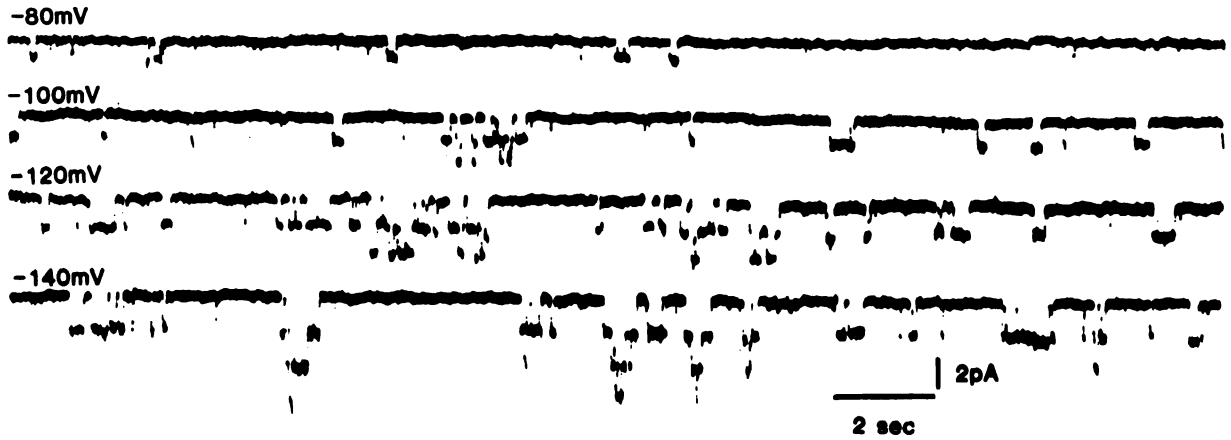

FIG. 1. *Voltage-dependent changes in ACh channel activities in an inside-out recording*

The micropipette solution contained 30 nM ACh. Mingograf records at −80 mV to −140 mV were obtained under control conditions. Amplitude of single-channel currents and number of channel openings increased at more negative membrane potentials. Bandwidth = 1 KHz.

TABLE 1

Voltage-dependent change of frequency in channel opening induced by ACh (30–200 nM) in cell-free patches (inside-out)

Cell no.	ACh concentration	Channel opening frequency[a] at membrane potential					
		−40 mV	−60 mV	−80 mV	−100 mV	−120 mV	−140 mV
	nM						
1	30	—	—	72%	100%	110%	135%
2	100	—	66%	78%	100%	—	—
3	100	30%	50%	76%	100%	—	—
4	150	33%	66%	62%	100%	—	—
5	150	34%	72%	78%	100%	—	—
6	200	—	69%	82%	100%	—	—
7	200	—	42%	81%	100%	—	—
Mean		32%	61%	76%	100%	110%[b]	135%[b]

[a] Expressed as percentage of frequency observed at −100 mV. The mean number of channel openings at −100 mV was 188 ± 44 events/min ($n = 7$).

[b] Determination made in one myoball.

gigaohm seal had been achieved) to both drugs. In addition to these changes, the appearance of a population of small-amplitude channels without many flickers was induced by Pyr (Fig. 2). The small-amplitude (1 pamp or less) channels were more prevalent at later stages of the recordings. At the beginning of the recording, shown in Fig. 2, there were many large-size channels (>2 pamp) similar to those seen under control conditions (Fig. 1). This number gradually decreased, and by 12–14 min after the gigaohm seal was achieved, fewer than 50% of the channel openings were of large amplitude. The number of low-conductance channels increased with time of exposure to Pyr (10–50 μM) and, eventually, if the concentration of Pyr was high enough, channel activity disappeared altogether or was markedly reduced. Another sample of ACh-induced channels altered by Pyr is illustrated in Fig. 3. In Fig. 3B and C, many flickers are apparent together with broadening of the baseline while the channel is open (see also Figs. 4 and 5). In fact, in the presence of Pyr (50–100 μM), the large number of flickers (Fig. 4B) apparently induced skewing to the left of the total amplitude histograms of single ACh and Pyr channel currents (Fig. 5B), a feature not seen when ACh was used alone in the patch pipette (Figs. 4A and 5A).

The histograms of ACh-induced single-channel open times in the presence and absence of Pyr, shown in Fig. 6, revealed a single-exponential distribution and a mean lifetime which was not altered, i.e., a mean value of 14.0 msec during control conditions to 14.6 msec during application of Pyr (50 μM) in combination with ACh (100 nM ACh) at −140-mV holding membrane potential (inside-out). Although the channel open times of the myoball illustrated in Fig. 6 were exponentially distributed, it should be mentioned that in some control recordings, particularly when the low-pass filter was set as high as 3 KHz, the histogram disclosed a double-exponential distribution. The fast component of this distribution usually occurred entirely within the first bin, that is, with lifetimes of 1–5 msec. This finding is consistent with other studies (3, 12). The effects of Pyr on ACh-induced single-channel currents, examined in cell-free patches at a holding potential of −100 mV, showed that Pyr (50 μM) produced no significant alteration of channel lifetime; i.e., in the presence of ACh alone in the micropipette the channel lifetime was 27.5 ± 2.9 msec (mean ± standard error, $n = 4$ myoballs), and 31.0 ± 2.6 msec ($n = 4$) when Pyr was together with ACh in the micropipette.

Alteration of ACh channels by Pyr applied in the bath

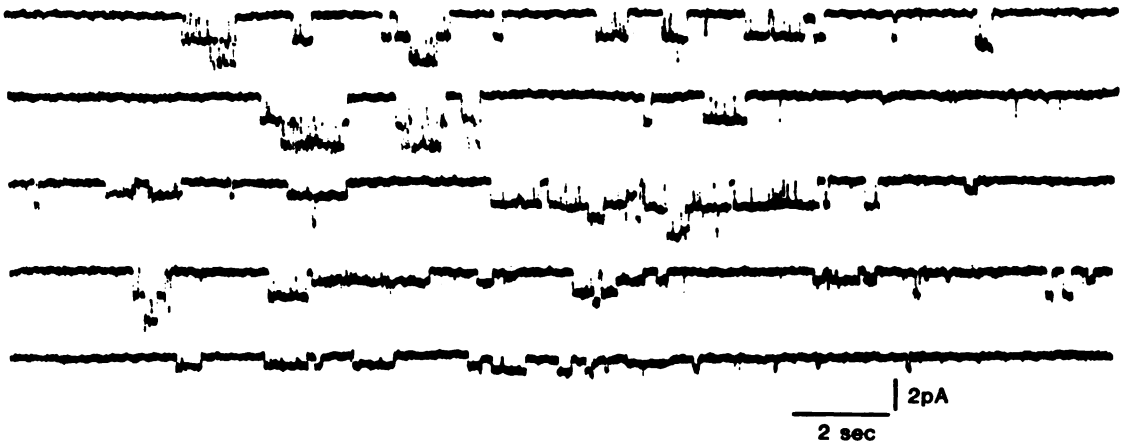

FIG. 2. *Effects of Pyr on ACh channels*

The micropipette solution contained 100 nM ACh and 50 μM Pyr. The holding membrane potential was +60 mV (pipette interior). Mingograf traces shown were recorded at various times after the establishment of a gigaohm seal: *top* and *second traces*, 2 min after the seal; *third* and *fourth traces*, 6 min; *bottom*, 10 min. Note that in *trace 3* low-conductance channels are present and by *trace 5* the channels are mostly with low conductance. Bandwidth = 1 KHz.

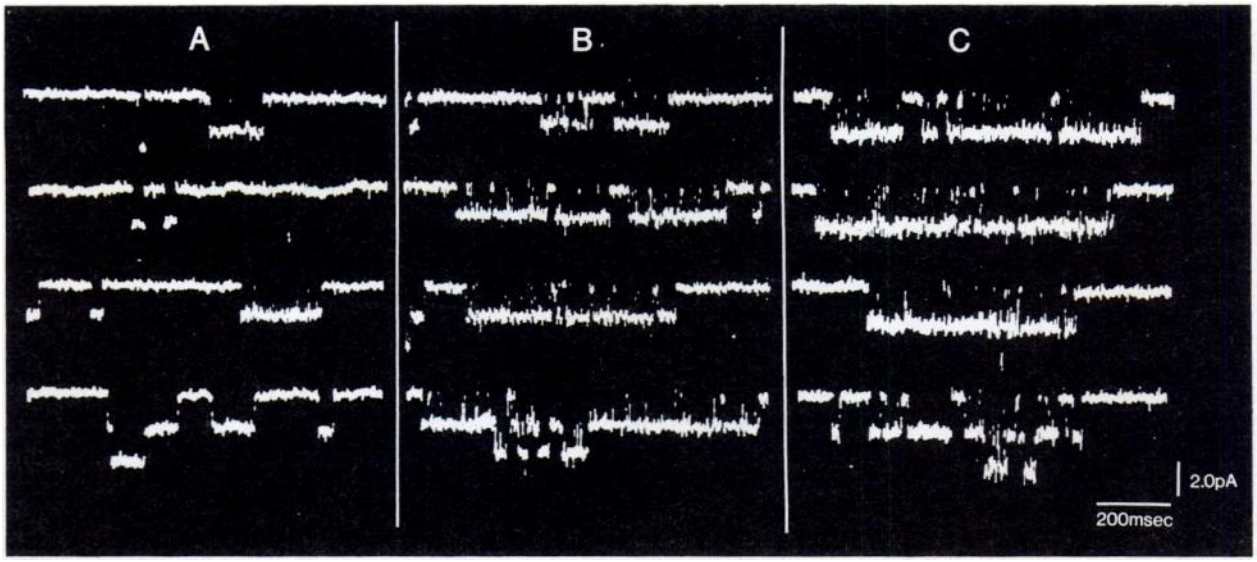

FIG. 3. *Samples of single ACh channels altered by Pyr*

Oscilloscope traces displaying single channels photographed to show the detail of channel openings. A, Control condition (ACh 100 nM alone in the micropipette) at +60 mV (pipette interior) on the myoball; B, 50 μM Pyr and 100 nM ACh were applied through the patch micropipette and currents were recorded at +60 mV (pipette interior) on the myoball; C, drug concentration was the same as in B, and currents were recorded at −100 mV inside-out. Bandwidth = 1 KHz. Note the marked increase in baseline width associated with flickering activities during channel opening at B and C.

(cell-attached patch). The amplitude of single ACh channel currents recorded in the myoballs was markedly altered following the superfusion of Pyr (50–100 μM) into the bath. This effect of Pyr was biphasic; i.e., the amplitude was initially increased to 120–130% of control values at 5 min after drug addition, followed by a marked decrease in the channel amplitude. The maximal depression of channel amplitude was obtained 30 min after the drug application. Total amplitude histograms showing the current-voltage (I–V) relationship for the same myoball under control conditions and after 30-min exposure to Pyr (50 μM or 100 μM) are given in Fig. 7. In the presence of 50 μM Pyr, reductions in channel amplitude to 86% of control at +80 mV (pipette interior) and to

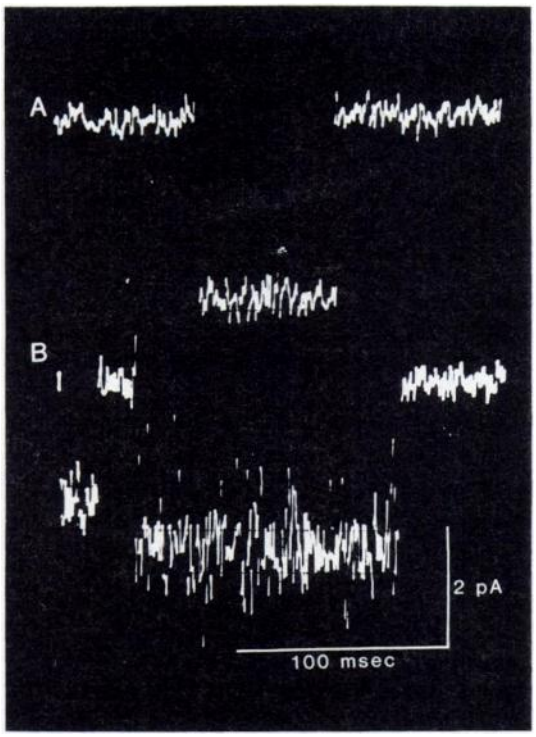

FIG. 4. *Oscilloscope traces of Pyr-induced baseline broadening during the open stage of a single ACh channel*

A, Control condition (100 nM ACh in the pipette) at +60 mV (pipette interior) on the myoball; B, 50 μM Pyr and 100 nM ACh were applied through the micropipette, and currents were recorded at +60 mV (pipette interior) on the myoball. Bandwidth = 1 KHz.

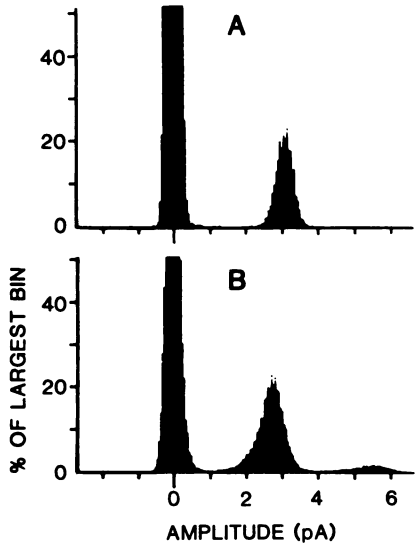

FIG. 5. *Total amplitude histograms of single-channel currents induced by ACh*

A, Control condition (100 nM ACh in the pipette) at −140 mV inside-out; B, 50 μM Pyr and 100 nM ACh were applied through the micropipette and currents were recorded at −140 mV inside-out. The first peak at 0 pamp shows the noise level of the closed-channel state. Subsequent peaks represent the noise level of the open-channel state.

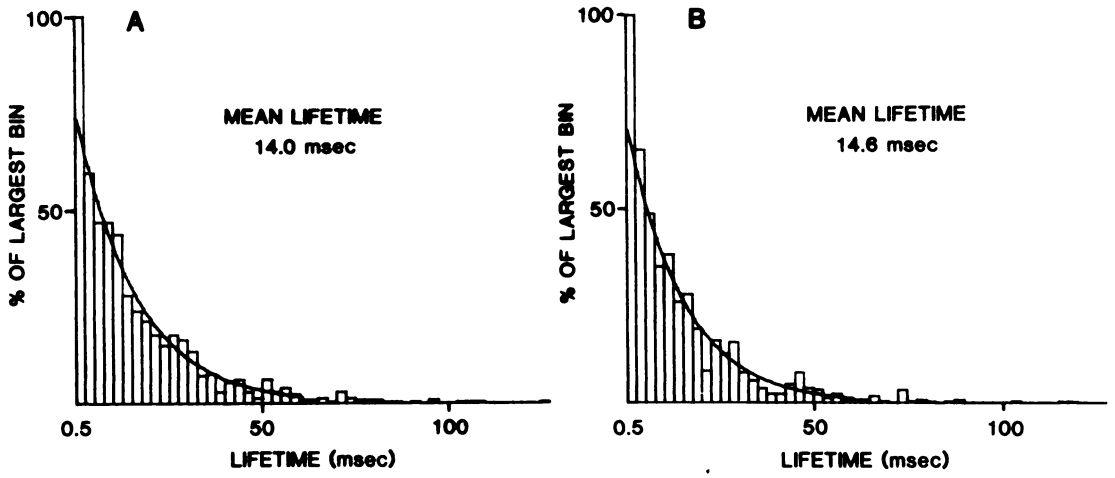

FIG. 6. *Histograms of single-channel open times*

Single-channel currents in a cell-free patch were recorded under control conditions (A) and in the presence of 50 μM Pyr (B). The holding membrane potential was −140 mV in both cases. The mean channel lifetimes were estimated from the regression line, in the presence and absence of Pyr.

67% at +60 mV were observed. At +40 mV, amplitude was reduced to a level which was indistinguishable from baseline. A further reduction was noticed with 100 μM Pyr. In this case the amplitude was reduced to 80% of control at +80 mV and 48% at +60 mV. As with 50 μM Pyr, the amplitude of single currents recorded at +40 mV was reduced to nearly baseline noise level (0.5 pamp) in the presence of Pyr (100 μM).

The effect of Pyr on the lifetime of the single-channel currents was also studied. In this series of experiments,

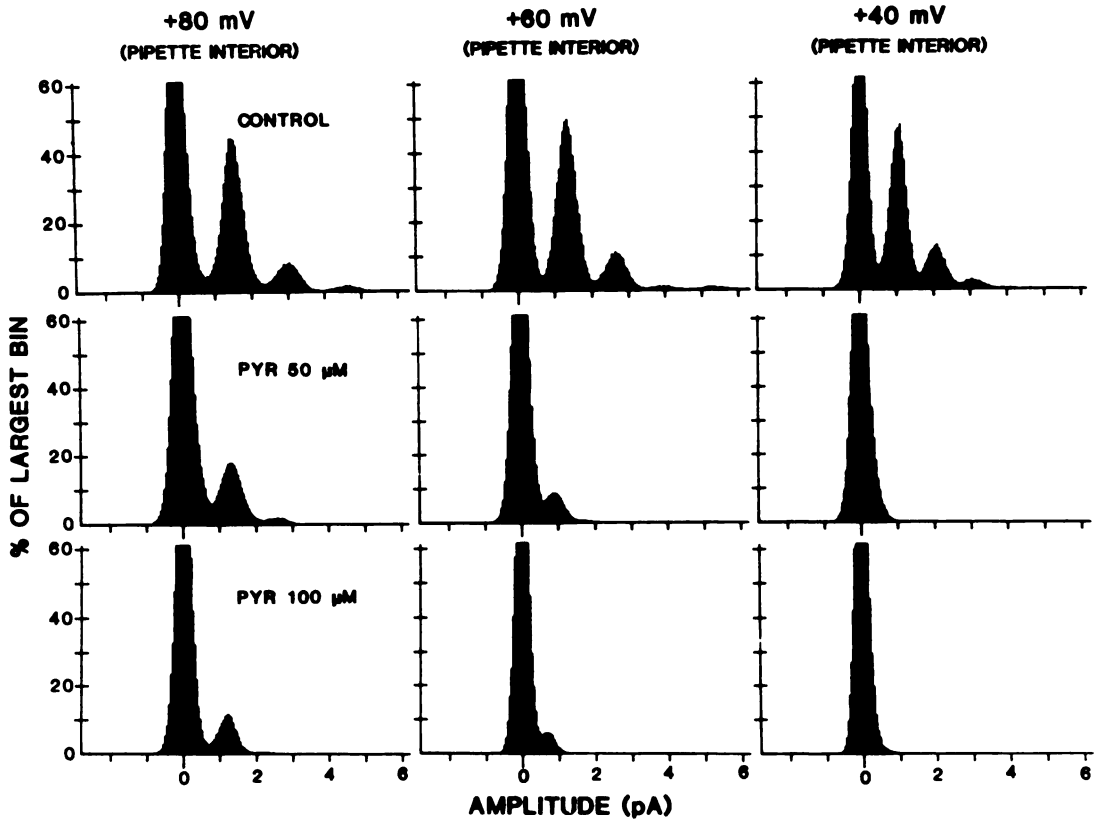

FIG. 7. *Total-amplitude histograms of single ACh channel currents under control conditions and in the presence of Pyr*

Data were obtained from one cell-attached patch at +80 mV (pipette interior) (*left*), +60 mV (*middle*) and +40 mV (*right*). The solution inside the micropipette contained 100 nM ACh, and Pyr was applied to the bath. *Upper row* shows control condition. *Middle* and *lower rows* present the data obtained 30–40 min after the application of two concentrations of Pyr. The abscissa in each histogram shows a current value [picoamps (*pA*)] converted from the difference between each point of digitized signals and baseline. The baseline (0 pamp on the abscissa) is represented by the first peak (the largest bin), or the noise level of the closed-channel state. Subsequent peaks represent multiples of unitary events (see Methods for details).

the mean lifetime at +80 mV (pipette interior) under control condition (25.8 ± 2.7 msec, $n = 3$) was unaltered by Pyr (50 μM; 26.6 ± 2.6 msec, $n = 3$). Subsequent application of a higher Pyr (100 μM) concentration induced a large number of fast flickerings. However, channel lifetime remained similar to control values (28.8 msec, $n = 2$) at 30 min after the drug application.

The frequency of channel openings induced by ACh was also affected by Pyr (Table 2). The control results were obtained from a patch in which the frequency of channel openings was constant at a high level (261 events/min; mean of three recordings). Pyr (50 μM) applied in the bath increased the frequency of channel openings to 112% of control values 5 min after the start of superfusion, followed by a decrease in frequency to 65% of control values at 30 min after the drug application. The frequency of channel openings was decreased to 38% of control values 30 min after the application of 100 μM Pyr.

Effect of Pyr on ACh channels recorded from cell-free patch (inside-out). Pyr applied in the bath produced results similar to those obtained when the drug was applied via the patch pipette. Pyr (50–100 μM) induced flickerings and marked enlargement of the baseline during channel open time (see Figs. 2–5). In addition, channels of amplitude similar to control conditions were gradually reduced in number, and were replaced by lower conductance channels in the presence of Pyr.

In the presence of Pyr (50–100 μM), a concentration-dependent reduction in the frequency of channel openings was observed at all the membrane potentials, while the voltage dependence was maintained (Fig. 8). A higher concentration of Pyr (200 μM) produced a biphasic effect on single channel activity. During the initial phase of drug application, the number of channel openings increased, and irregular waves of bursting activity were seen (Fig. 9). After the cessation of the bursting activity, channel opening was markedly reduced. Figure 10 shows a sample of the I–V relationship in one cell-free patch. The conductance of ionic channels under control conditions was 19.5 pS and 12.3 pS in the presence of Pyr (50 μM). The reversal potential extrapolated from the I–V plot was 0 mV.

Agonist effect of Pyr on the rat myoballs. When the patch electrodes were filled with a solution containing Pyr (50–100 μM) alone (i.e., without ACh), low-frequency channel openings with conductances in the range of 1–1.5 pamp at +100 mV (pipette interior) were recorded in the cell-attached patch (Fig. 11). The amplitude of the Pyr-induced channel currents was voltage-dependent (Table 3). The channel conductance, estimated from the I–V relationship, was 11.7 pS and the reversal potential was 0 mV. When applied directly on the myoball 1–10 min after the gigaohm seal was achieved, the frequency of channel openings induced by Pyr (100 μM) recorded at −80 mV was 5.6 ± 1.5 events min^{-1} (mean ± standard error; $n = 5$ myoballs). The values for channel lifetime induced by Pyr are shown in Table 3.

Channel openings in the presence of Pyr alone (50–100 μM) were observed 1–30 min after the establishment of gigaohm seals, but the frequency was low. At a higher concentration (1 mM), Pyr channel openings similar to those seen with 50–100 μM drug were also observed with low frequency and often with bursts of channel activity followed by long periods of silence. These channel open-

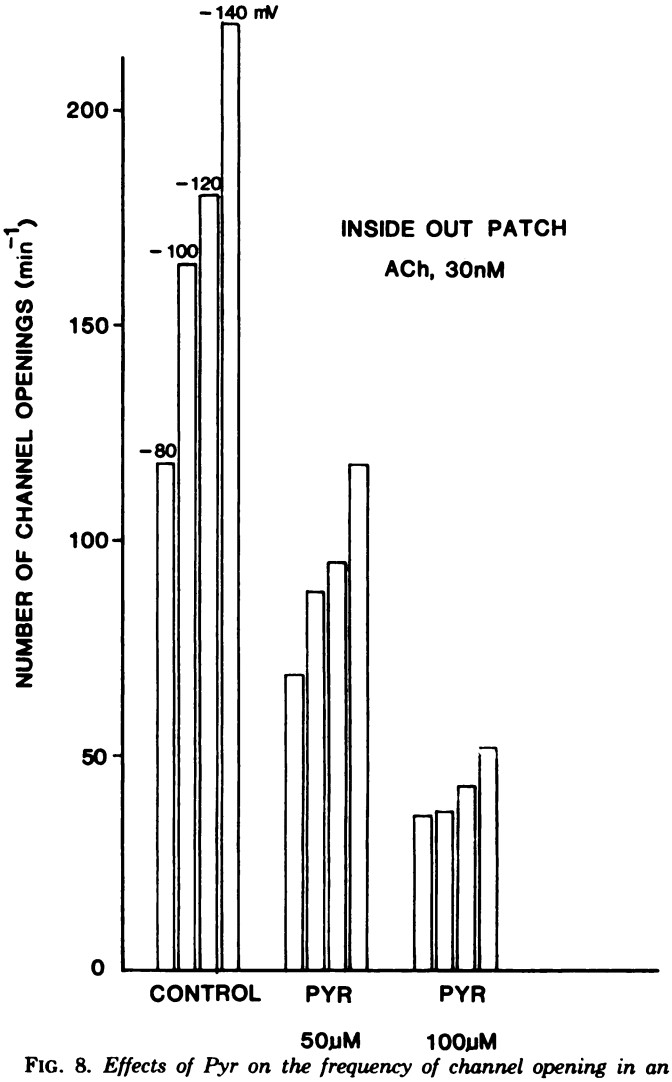

FIG. 8. *Effects of Pyr on the frequency of channel opening in an inside-out patch*

The micropipette solution contained 30 nM ACh. Pyr (50–100 μM) was applied to the bath. The number of channel openings under each condition was counted for 4 min of continuous recordings and expressed as events per minute. An important feature of this figure is that the voltage dependence of ionic channel opening frequency was maintained during drug action.

TABLE 2

Effects of Pyr on frequency of channel openings induced by ACh and recorded in cell-attached patches (on the myoball)

Channel openings were counted during 5-min intervals. Single-channel currents were recorded at a holding potential of +80 mV (pipette interior).

Cell no.	Control	Pyr, 50 μM		Pyr, 100 μM	
		5 min	30 min	5 min	30 min
1	100% (361)[a]	105%	60%	—	43%
2	100% (303)[a]	111%	77%	44%	38%
3	100% (118)[a]	121%	59%	46%	32%
Mean	100% (261)[a]	112%	65%	45%	38%

[a] Number of channel opening events per minute.

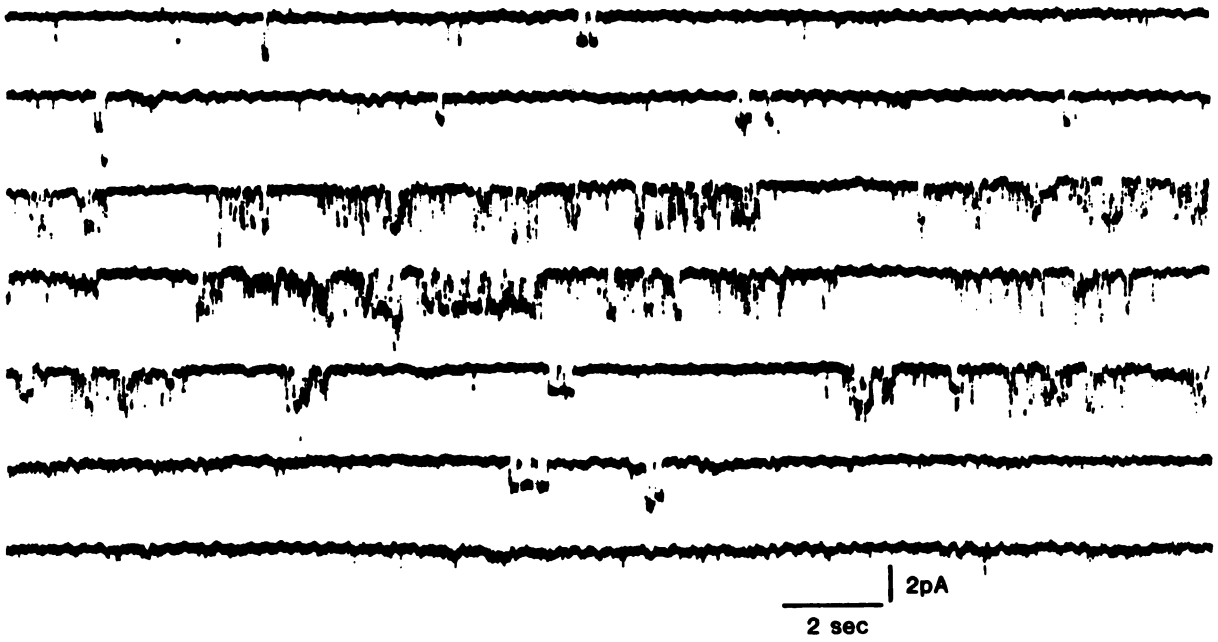

FIG. 9. *Effects of higher concentration (200 μM) of Pyr on ACh (30 nM) channel activities*
The single-channel currents at −100 mV were recorded in the same inside-out patch shown in Fig. 1. Pyr was applied to the bath. *Upper four* Mingograf traces represent the continuous recording between 10 and 11 min after the application of the drug. *Lower three traces* show the recording 15 min after the application. Note the marked burst of channel activity followed by complete cessation of channel openings. Bandwidth = 1 KHz.

ings at 1 mM Pyr disappeared 10–15 min after the establishment of the gigaohm seal.

To test whether or not the channel openings induced by Pyr were the result of an interaction of the drug with nicotinic receptors on the myoball, experiments were performed using pretreatment of the myoball with α-BGT in suitable concentrations to block initially the ACh receptor. When the myoballs were pretreated with α-BGT (5 μg/ml) for 30 min, no channel openings were observed with Pyr (50 μM–1 mM) alone, ACh (300 nM) alone, or the combination of both drugs in the micropipettes.

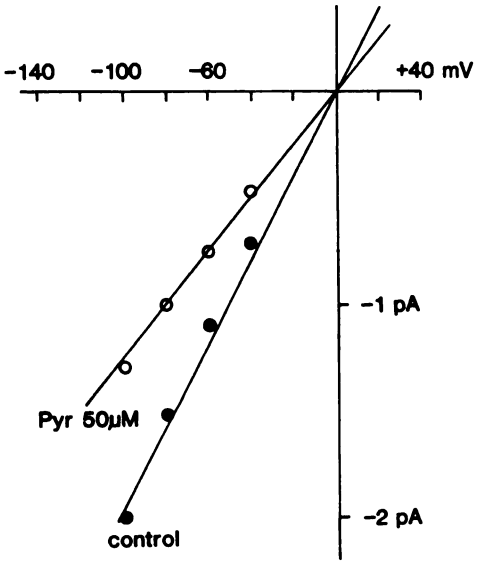

FIG. 10. *Current-voltage relationship of single ACh channels of an inside-out patch*
● and ○, Control and 50 μM Pyr, respectively. The micropipette solution contained 100 nM ACh, and Pyr was applied to the bath. Abscissa: membrane potential (millivolts). Ordinate: amplitude of single-channel currents (picoamps) estimated from the total-amplitude histograms.

To test whether or not this weak agonistic action of Pyr on the rat myoball could also be seen on mature muscles, we have dissected single fibers of the interosseal muscles of the frog toe and removed the connective tissue with collagenase so that gigaohm seals with the patch pipette could easily be achieved at the perisynaptic region.[5] Preliminary studies using these single muscle fibers disclosed effects of Pyr similar to those seen on the myoball. Upon obtaining a gigaohm seal with a micropipette containing Pyr (100–200 μM) alone, low-conductance channels occurring at low frequencies were observed having characteristics similar to those recorded from the myoball. These channel openings were not observed after pretreatment with α-BGT.[5]

DISCUSSION

The results of the patch clamp study showed that Pyr decreased both the amplitude of ACh-induced single-channel currents and the frequency of channel openings, without changing the mean channel lifetime. In addition, Pyr alone opened channels by itself as a weak agonist ($Pyr + R \rightleftharpoons PyrR^*$). The low-conductance, low-frequency channels induced by Pyr alone resemble the altered channels that become predominant after prolonged exposure to ACh plus Pyr (see *bottom trace* in Fig. 2). Pyr caused marked alterations of the ACh-induced channel, including intensive flickering and widening of the baseline during open channels followed by nearly silent periods (see Figs. 4 and 9). These phenomena, character-

[5] A. Akaike and E. X. Albuquerque, unpublished results.

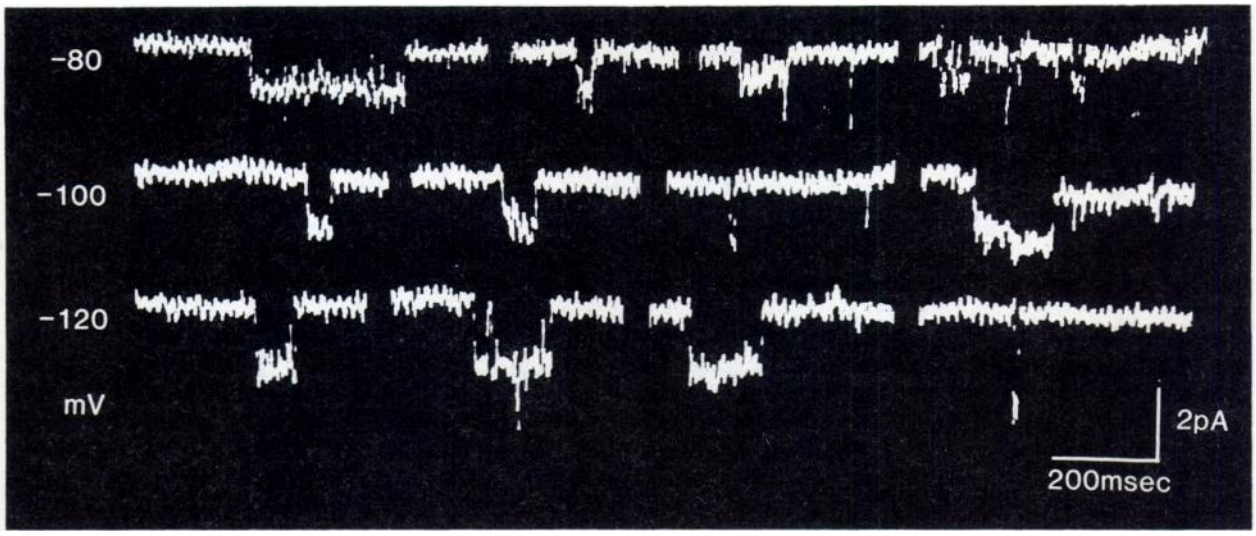

FIG. 11. *Samples of single channels induced by Pyr as an agonist*
The micropipette solution contained Pyr alone (100 μM). The recordings are from the cell-attached patches at holding membrane potentials of +80 to +120 mV (pipette interior). Bandwidth = 1 KHz.

istics of Pyr action, may indicate the appearance of desensitized receptor-ionic channel complexes (13). Anticholinesterase effects are not involved since the myoballs, after washing with the Hanks' BSS, had extremely low levels of the enzyme (14).

Pyr, which is a quaternary amine, had similar effects whether it was applied to the myoball via the patch pipette or via the bathing medium either under cell-attached or "inside-out" recording conditions. This suggests that the drug would have access to the receptor surface through the micropipette-membrane seal, through the ACh channel itself, or through the lipid phase of the membrane. The possibility that Pyr could have reached the inside of the patch pipette via the gigaohm seal formed between the micropipette and the surface of the myoball or muscle membranes is most unlikely. If such were the case, it would be difficult to explain the observation that other quaternary agents, such as QX314 (15), tetraethylammonium[6] (10, 16), and meproadifen (17–19), have effects on the receptor channel molecule only when they reach the outer surface of the membrane through the pipette. In addition, pipette seal resistances on the order of 10 gigaohm are consistent with glass-membrane separations of about 1 Å (4). The access of the Pyr molecule to the receptor sites on the outer surface via the pore of the ionic channel itself also seems improbable. If we assume that the diameter of the open pore of the ACh channel is about 6.5 × 6.5 Å (20–22), an elongated molecule like Pyr, with a diameter of 6.9 Å measured at its narrowest point (from Van der Waals distance at the hydrogen located at position 2 to hydrogen on position 5) using a Cory-Pauling-Koltum model, would have great difficulty traversing the lumen of the channel. For Pyr applied in the bath in the cell-attached condition to gain access to the extracellular surface of the membrane patch under the micropipette via channels, it would have to gain access to the cell interior and subsequently to exit the cell into the micropipette. A more likely possibility is that Pyr diffuses through the lipid phase. Some indirect lines of evidence support such a hypothesis. For example, one is that Pyr is slowly reversible upon washout; i.e., only upon a 60-min wash was a partial recovery of neuromuscular transmission observed (see Fig. 2 and ref. 6). Accordingly, Pyr may be able to diffuse into the lipid phase and possibly with lateral diffusion gain access to the extracellular surface of the membrane beneath the micropipette. Thus, the drug could eventually produce effects under all conditions of application in a given period of time. In fact, with the drug inside the patch pipette, the effect is immediate; application of Pyr via the bath under cell-attached or "inside-out" conditions yields a similar qual-

[6] Y. Aracava and E. X. Albuquerque, unpublished results.

TABLE 3

Amplitude and lifetime of single-channel currents induced by Pyr

The micropipette solution contained Pyr (50–100 μM) without ACh. Data were obtained from five cell-attached patches and two inside-out patches. Each value (channel amplitude and lifetime) is the mean of 20–30 events per patch. These data were analyzed by hand because of the low frequency of single-channel events.

Holding membrane potential	On the myoball				Inside-out		
	−60 mV	−80 mV	−100 mV	−120 mV	−60 mV	−80 mV	−100 mV
Amplitude (pamp)	1.02	1.24	1.48	1.69	0.69	0.94	1.14
Mean lifetime (msec)	16.8	26.5	27.5	31.0	9.9	15.4	18.9

itative result but with a predictable delay in the case of the cell-attached condition. This would suggest that in preparations other than the myoball, Pyr may penetrate the intracellular compartment of the cell, and may have access to the central nervous system as well. Birtley *et al.* (23) demonstrated that Pyr, when systemically applied, accumulated in skeletal muscles at a concentration 3–4 times greater than that in blood. Accumulation of Pyr in muscle indicates that the drug can penetrate into the muscle membrane, even though Pyr is a quaternary compound.

In the presence of Pyr at concentrations of 50–100 μM the frequency of channel opening events induced by ACh recorded in the myoball was initially increased and then progressively decreased and eventually abolished. Indeed, at 200 μM concentrations of Pyr, there was an initial increase of the channel openings with marked burstlike activity, followed by a fast reduction of channel activity. These observations suggest that enhancement of affinity of ACh to the receptor site produced by Pyr (6) may accelerate the desensitization of the ACh-ionic channel complex. Although a large number of channel openings in the presence of Pyr and ACh together appeared longer than that under control conditions (see Figs. 4 and 5), these channel openings were frequently interrupted by flickers. The analysis of channel lifetime showed negligible change from control conditions (filter bandwidth 1 KHz, and the flickers within open channels were counted as channel closures; for details see Methods). This may appear to conflict with the finding reported in the previous paper (6) that channel lifetime as revealed by noise analysis was prolonged. A possible explanation for this discrepancy is that the fast-flickering events do not contribute sufficient power to the spectra to be detected in noise analysis. Neher and Steinbach (24) reported that the lidocaine derivative QX-222 produced flickers during ACh open channels and shortened the lifetime of these channels. The local anesthetic agent bupivacaine also shortened channel lifetime but without inducing flickering (25). It is unlikely that flickering observed in the presence of Pyr is due to channel blockade as seen with some local anesthetics, since Pyr did not change the channel lifetime even when flickering was observed. In addition, Pyr during its effect on the ionic channel opened by ACh caused a marked broadening in the width of the channel baseline (see Figs. 3 and 4). This broadening of the baseline observed during channel opening may reflect a very fast rate of flickering which cannot be resolved completely during our recording, even using a 3-KHz bandwidth filter. Such a phenomenon is also seen with meproadifen (19). This agent, which increases the affinity of the agonist for the ACh receptor to a greater extent than does Pyr itself (17–19), does not change channel conductance or lifetime. Apparently, meproadifen and Pyr, by enhancing the affinity of ACh for its receptor, may cause an increase in receptor activation, as evidenced by an increase in an initial frequency of channel opening followed by desensitization. Thus, one can hypothesize that Pyr-induced broadening of the baseline during channel opening reflects its ability to increase affinity of the agonist to its binding site. Such an effect may be related to perturbations of the conformations of the receptor-channel macromolecule occurring during channel activation (13), a phenomenon which is not clear at the present moment.

In addition to these alterations of ACh channel currents, when Pyr was applied in the patch pipette at concentrations of 50 μM–1 mM without any other nicotinic agonists present, we observed channel openings of low conductance. The frequency of these channel openings was very low even at these high concentrations. Channel currents induced by Pyr were voltage dependent and were blocked by pretreatment with α-BGT, a nicotinic receptor antagonist. The sodium channels were blocked in our experiments by pretreatment with tetrodotoxin. Although potassium channels were intact in the present experiments, it is most unlikely that potassium channels were related to the single-channel currents produced by Pyr since the polarity of Pyr-induced channel currents at the ranges of potentials studied would be opposite to that expected for potassium channel currents (26, 27). Since α-BGT blocked the single currents induced by Pyr, one may conclude that Pyr behaves as a weak agonist of the nicotinic receptor in the rat myoball and the perisynaptic region of the frog interosseal muscles. The low value for conductance of channels which are opened by Pyr alone in myoballs and also in the perisynaptic region of single fibers of the interosseal muscle[5] is consistent with previous observations by Spivak *et al.* (28) that agonists of the nicotinic receptor can induce channel openings displaying different conductances. It has also been shown that conductance is not necessarily correlated with agonist potency (28). Therefore, the low frequency of channel openings induced by Pyr may be related to its low potency. The appearance of single channels with low conductance in the presence of Pyr and ACh may be partly explained by the weak agonist property of Pyr. However, since the opening frequency of low-conductance channels in the presence of the combination of Pyr and ACh was higher than that induced by Pyr alone, it is unlikely that the competition of Pyr and ACh for the same binding sites could account entirely for the occurrence of these channels. It is more likely that, in addition to this agonistic effect, Pyr allosterically modifies the nicotinic receptor-ion channel complex, inducing alteration of the conductance properties of the ACh-activated channels.

In summary, this study has shown that Pyr has a direct interaction with the receptor site of the nicotinic ACh receptor-ion channel complex. The agent in combination with ACh causes the appearance of channels of normal amplitude with fast flickering and an enlargement of the baseline during the open state. These large, flickering channels may represent a conformational state which eventually will yield the formation of a low-conductance and low-frequency species. The final development of this feature is a complete cessation of channel opening, a state which resembles that induced by ligands which increase the affinity of the receptor for its active sites (17–19). In addition, Pyr alone was able to open channels with low-conductance and low-frequency characteristics. These results, which are consistent with the

findings in the accompanying paper (6), provide new evidence for the actions of this agent at sites on the ACh receptor-ion channel complex in addition to its anticholinesterase activity.

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