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# Effects of Irreversible and Reversible Cholinesterase Inhibitors on Single Acetylcholine-Activated Channels

Robert Zorec, Maria Scuka†, and Marjan Kordaš
Institute of Pathophysiology, Medical School, University of Ljubljana,
61105 Ljubljana, Slovenia, (YU), and †Department of Biology, University of Trieste, Trieste 34127, Italy

**Summary.** The use of cholinesterase (CHE) inhibitors provided valuable information about the mechanism(s) of neuromuscular transmission, but questions on side effects at the level of ACh-activated channels were raised. Patch-clamp recording was used to study the effects of prostigmine (PST) and methanesulfonyl fluoride (MSF), a reversible and an irreversible cholinesterase inhibitor, respectively, on ACh-activated channels. We found that these drugs diminish the average dwell time of elementary currents from around 5 msec (control) to less than 1 msec in the presence of PST (20 $\mu$M) or MSF (5 mM) (at room temperature). With MSF the ACh-activated channel conductance of the most frequently observed amplitude class decreased from 45 pS (control) to 30 pS, but not in the presence of PST. In control conditions there were also amplitude classes of 60 and 24 pS, with probabilities of occurrence <10%. In the presence of 1.5 mM MSF, where current dwell time was not affected, additional subconductance states of 19 and 36 pS were observed and may be due to partial blockade of the open channel. We conclude that the drug of choice to be used in studies on the role of CHE in the neuromuscular transmission is MSF, because at 20 $\mu$M PST, where blockade of ACh-activated channels is significant, cholinesterase was reported to be partially inhibited, whereas at 1 mM MSF it is fully inhibited and the dwell time of ACh-activated channels is not affected.

**Key Words** single-channel recording · adult rat skeletal muscle · cell culture · cholinesterase · acetylcholine/receptor channel · anticholinesterase drugs

## Introduction

At the neuromuscular junction the action of acetylcholine (ACh) must be short to effectively depolarize the membrane, to evoke the action potential, and consequently, the muscle fiber contraction. Cholinesterase, an enzyme resident in the cleft of the synapse, rapidly hydrolyzes ACh and is believed to influence the kinetics of the neuromuscular transmission, because it removes the released neurotransmitter from the cleft. With this the transmitter action on the postsynaptic receptor channels is shortened.

The most common approach to study the role of cholinesterase on neuromuscular transmission is by using various anticholinesterase drugs. Although such experiments have provided important information about the mechanism of neuromuscular transmission, they also raised notes of caution. It appeared that anticholinesterase drugs may not act solely on the enzyme. In the very early studies (Eccles & MacFarlane, 1949; Del Castillo & Katz, 1957) actions on ACh receptors were proposed. These drugs, at high concentrations, while prolonging the decay of end-plate currents (Kordaš, 1972*a*,*b*; Magleby & Stevens, 1972*a*,*b*; Pascuzzo et al., 1984), also reduced the amplitude of end-plate potentials and currents (Eccles & MacFarlane, 1949; Kuba & Tomita, 1971; Kordaš, Brzin & Majcen, 1975). A decrease in the amplitude of the end-plate responses, which appeared after a transient increase, was also observed at relatively low concentrations of anticholinesterases (Scuka, 1987, 1988; Deana & Scuka, 1990).

The nature of the interaction(s) of the cholinesterase inhibitors with ACh receptors-channels was reported to involve weak agonist properties, block of the open channel, desensitization, and altered channel conductance properties (Kuba et al., 1974; Katz & Miledi, 1977; Akaike et al., 1984; Shaw et al., 1985; Albuquerque et al., 1988).

In this paper, we examined the effects of prostigmine (PST) and methanesulfonyl fluoride (MSF) on the properties of single ACh-activated elementary currents recorded in long-term cultured adult muscle fibers. These two drugs were selected because of their different chemical structure and because PST is a reversible cholinesterase inhibitor whereas the action of MSF is an irreversible agent. The purpose of the study was to search for possible different effects of these two drugs on ACh-activated elementary currents.

## Materials and Methods

### Cell Culture

Experiments were carried out on adult muscle fibers (from Wistar rats) prepared as a suspension in culture. Single muscle fibers were obtained from the *M. flexor digitorum brevis* according to a modification of the method of Bekoff and Betz (1977). Briefly, the muscles were dissected from the animal and placed in 35-mm culture dishes, in 2 ml Eagle's Minimal Essential Medium (MEM; Flow, UK) supplemented with horse serum (5% by volume; Flow), penicillin (100 units $ml^{-1}$), streptomycin (100 $\mu g\ ml^{-1}$) and 2 mM L-glutamine. To this medium, collagenase (GIBCO) was added (3 mg $ml^{-1}$). The muscles were incubated for 3 hr at 36°C in an air-5% $CO_2$ incubator at 95% humidity, washed in fresh EMEM and mechanically dissociated into single muscle fibers by repeated passages through Pasteur pipettes, the tips of which had been fire polished. The isolated muscle fibers were cultured in plastic dishes for up to two weeks. Cultures of five to nine days were used in the experiments.

### Single-Channel Recording

Previous to the experiment the fibers were transferred into the recording chamber and bathing solution of the following composition (in mM): 131.8 NaCl, 5 KCl, 2 $MgCl_2$, 0.5 $NaH_2PO_4$, 5 $NaHCO_3$, 10 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 10 D-glucose, 1.8 $CaCl_2$, pH 7.2 (NaOH). The chamber was mounted on the base of an inverted OPTON IM 35 microscope, and the preparation was visualized at 200 to 400 magnification with phase-contrast optics. All experiments were carried out at room temperature.

Recording pipettes were pulled from borosilicate capillaries (GC120F, Clark Electromedical Instruments, Reading, UK) on a vertical puller (Palmer BioScience, UK), with a diameter of about 1–2 $\mu$m (resistances in the range 10–20 MΩ) and heat polished according to the methods of Corey and Stevens (1983). Pipettes were filled with 100 to 200 nM ACh (Sigma) dissolved in the bathing solution. Prostigmine (neostigmine methyl sulfate, Sigma) and methanesulfonyl fluoride (Eastman Kodak) were added to this solution at concentrations stated. Gigaohm-seal, cell-attached patches from the middle part of the fiber were obtained with conventional techniques (Hamill et al., 1981) with a home-made amplifier (Henigman, Kordaš & Zorec, 1987; *see also* Zorec et al., 1991).

The current signal from the amplifier was stored on a VHS video recorder (Tensai) following pulse-code modulation (modified 501ES, Sony; Lamb, 1985) for subsequent analysis. Signals were low-pass filtered at 3 kHz (−3 dB, 4-pole Bessel) and connected to the analog-to-digital converter CED 1401 (Cambridge, UK), sampled at 10–20 kHz and stored on a hard disk (IBM PC compatible). Acquisition and analysis software was kindly provided by Mr. John Dempster (Strathclyde University, Glasgow, UK).

Single-channel currents were analyzed by using the program described previously (Dempster, 1989). The current amplitude distribution was determined to obtain the mean current amplitude value at different pipette potentials. In general each histogram had at least two peaks corresponding to baseline noise and open state of the ACh-activated elementary currents. Amplitude distributions were formed from measurements of average amplitude of each elementary current event and displayed as percentage of states per current interval. In some cases mean amplitude of elementary currents were also determined by measurements (5 to 30 events) directly from the screen of the computer monitor by using the cursors of the program. Where estimates of the mean current levels obtained by measurements of only some events (main and subconductance states) were compared with means obtained by automatic computer measurements, the difference between the two values was less than 5%.

The amplitude distributions determined by automated method were well fitted by Gaussian curves. In some experiments a slight skewness of the elementary current amplitude distribution of the ACh-activated elementary current events was observed. These humps in the shape of the distribution corresponded to additional classes of ACh-activated elementary currents. The amplitudes of these events were measured manually by inspecting the whole signals in which skewness of the open-time amplitude distribution were observed. Where subconductance states were more frequent, the amplitudes of digitized elementary currents were arbitrarily divided into two or three groups by means of a threshold interval (50%, 15 to 85%, 25 to 75%, 30 to 70% of the mean amplitude). This improved the current amplitude resolution within the selected range of current amplitude values. During such procedures signals were also low-pass filtered (1 kHz, −3 dB, Bessel).

Channel conductance was determined from the slope of the current *versus* voltage relation obtained by linear regression. In the case of few subconductance states, channel conductance was estimated from the amplitude of the elementary currents and the driving potential, which was taken to be equal to the sum of pipette holding potential and the estimated reversal potential for the current-voltage relation of the main conductance state. A reversal potential of 0 mV for ACh-activated elementary currents was assumed (Mishina et al., 1986).

For kinetic analysis the same digitized records were used, after reduction to a table of transition times and amplitudes by a routine of amplitude threshold analysis. The signal was scrolled until an amplitude greater than the threshold (30 to 50% of mean elementary current amplitude) was detected. The open time was defined as the duration of the event at one-third to one-half the mean amplitude current. A histogram was formed and an exponential distribution curve was fitted to the data, composed of one, or the sum of two, exponential functions. The minimum duration accepted in this analysis was 150 $\mu$sec (20-kHz sampling rate) and 300 msec (10-kHz sampling rate). Existence of additional conductance states was usually not taken into account in such elementary current kinetic analysis, as the probability of observing these states was typically around 10% of all elementary currents. This procedure used all data points within the elementary current event.

## Results

### Comparison of Kinetic Properties of ACh-Activated Elementary Currents in Control Conditions and in the Presence of PST and MSF

In denervated muscle fibers ACh-activated channels are characterized by a longer dwell time and a smaller conductance (Katz & Miledi, 1972; *see* Schuetze & Role, 1987; Brehm & Henderson, 1988;

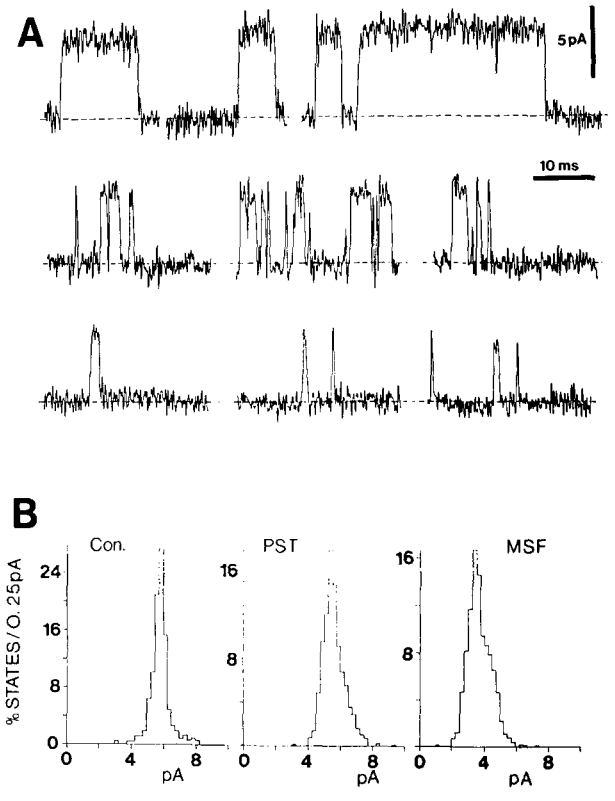


**Fig. 1.** (*A*) Properties of ACh-activated elementary currents in the presence of anticholinesterase drugs. Patch pipette contained 200 nM ACh, to which PST (prostigmine, 20 $\mu$M, middle trace) or MSF (methanesulfonyl fluoride, 5 mM, lower trace) was added. Top trace shows elementary currents (upward deflection denotes current of cations into the cytosol here and elsewhere in the paper) in control conditions (pipette potential 88 mV), middle trace when PST was added (pipette potential 81 mV), and lower trace in the presence of MSF (pipette potential 84 mV). Note the reduction in elementary current dwell time, while elementary current amplitude is similar in all three membrane patches. (*B*) Amplitude distributions of elementary current events from recordings of *A*. Amplitude distributions of the recordings in the middle trace (5.6 pA, 446 events), top trace (5.9 pA, 1253 events) and bottom trace (3.9 pA, 711 events) are shown. Amplitude distributions were obtained from average elementary current amplitudes (*see* Materials and Methods)

*see also* Steinbach, 1989). It was reported that adult muscle fibers cultured for more than four days acquire properties which are similar to those obtained in 'clasically' (*in situ*) denervated muscle fibers (Ruzzier & Zorec, 1988; F. Ruzzier, F. Grohovaz, P. Lorenzon, and R. Zorec, *submitted*). We have used the same preparation for the study of drug single-channel interactions, because the elementary current dwell time is not likely to be limited by the bandwidth of the recording system.

Figure 1*A* (upper trace) shows ACh-activated elementary currents which have three to four times longer average dwell times than the ACh-activated channels in nondenervated muscle fibers (junctional type) (Brehm & Henderson, 1988; Ruzzier et al., *submitted*). The distribution of elementary current dwell times was best fitted by the sum of two exponential functions with time constants which were around 0.5 and 7 msec, as shown in Fig. 2 left (average dwell time 4.9 msec, 634 events, pipette potential 88 mV). These results are in agreement with previous reports (Henderson, Lechleiter & Brehm, 1987). In four out of five experiments we have also seen additional conductance states (one smaller and one higher, *see* Fig. 4), where the probability of occurrence was 10.0 ± 8.8% (mean ± SD, $n$ = 4), and these events were therefore not excluded from the kinetic analysis in these experimental conditions.

The middle and lower traces of Fig. 1*A* show the effects of the presence of PST (20 $\mu$M) and MSF (5 mM) in the patch pipette, respectively, on ACh-activated elementary currents. In previous experiments it was shown that these concentrations inhibit the activity of cholinesterase (Kordaš et al., 1975). It is clear that the dwell time of elementary currents has decreased considerably. Figure 2 shows this effect quantitatively, where dwell-time distributions were best fitted by one exponential function with time constants of 0.9 and 0.4 msec, for PST and MSF treatments, respectively. Similar results were obtained in four other patches with PST (20 $\mu$M) and MSF (5 mM).

A smaller reduction in dwell time was also observed with 6 $\mu$M PST ($n$ = 2). In one of these, at 82-mV pipette potential, average dwell time was 2.1 msec (0.6- and 2.3-msec time constants, 485 events) and 0.5 msec (0.2- and 2.2-msec time constants, 405 events). However, with 1.5 mM MSF no reduction of average dwell time was observed. At 84-mV pipette potential (similar conditions as in experiment of Figs. 1 and 2), average dwell time was 5.5 msec (0.3- and 6.3-msec time constants, 682 events).

Similar results were obtained in experiments ($n$ = 5) with 100 nM ACh in the pipette. In control conditions no apparent desensitization was observed.

## Relationship between Changes of the Kinetic Properties of ACh-Activated Elementary Currents by PST and MSF, and Channel Conductance

Elementary current amplitudes were normally distributed (Fig. 1*B*, *see also* Figs. 4 and 5). The amplitude of the events in the middle trace of Fig. 1*A* (*see* Fig. 1*B*, legend) is slightly smaller than the events on the top trace (Fig. 1*A*, *see* legend to the figure).

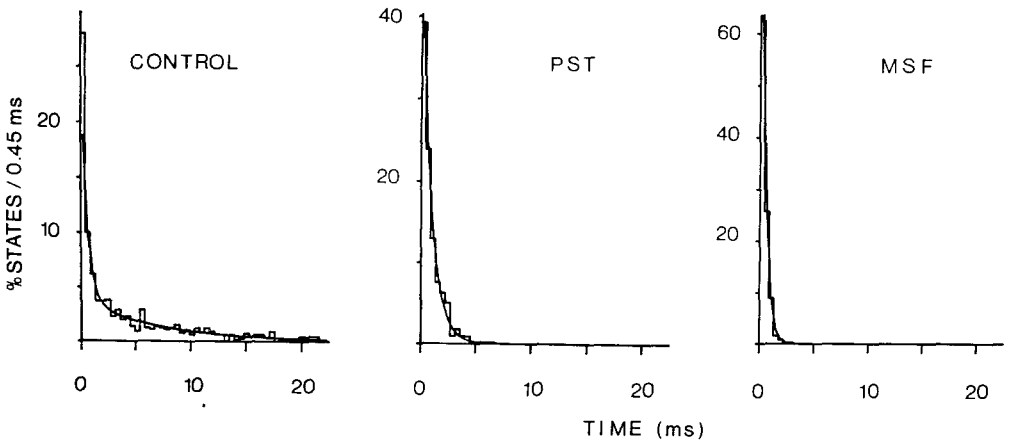


**Fig. 2.** Open dwell-time distributions for elementary ACh-activated currents recorded in control conditions (right), in the presence of PST (20 $\mu$M, middle) and in the presence of MSF (5 mM, left). In control conditions the distribution was best fitted by a sum of two exponential functions with time constants of 0.5 and 7.0 msec (634 events), whereas in conditions where PST or MSF as added to the pipette solution the distributions were best fitted by one exponential with significantly smaller time constants of 0.9 and 0.4 msec, respectively. (Middle histogram was obtained from 558 events and the right one from 392 elementary currents.)

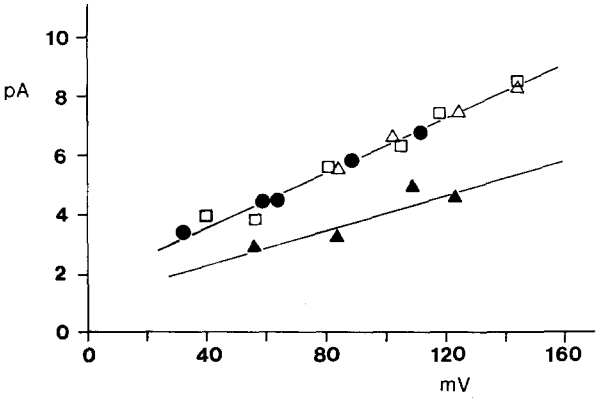


**Fig. 3.** The effect of PST (20 $\mu$M, open squares) and MSF (1.5 mM, open triangles; 5 mM, filled triangles) on the dependence of the ACh-activated elementary currents (most frequently observed) *versus* pipette potential (filled circles, control). Conductances for each experiment were determined separately by linear regression of the following forms: $y_1 = 0.0436 \cdot x_1 + 1.810$ (control, corr. = 0.99, $n = 5$), $y_2 = 0.0463 \cdot x_2 + 1.690$ (PST 20 $\mu$M, corr. = 0.96, $n = 6$), $y_3 = 0.0445 \cdot x_3 + 1.950$ (MSF 1.5 mM, corr. 0.99, $n = 4$) and $y_4 = 0.0297 \cdot x_4 + 1.120$ (MSF 5 mM, corr. = 0.91, $n = 4$), where $y$ and $x$ values are in pA and mV, respectively, in these and other regression procedures elsewhere in the paper. Note that each current *vs.* voltage relation was normalized against the reversal potential of the control experiment by shifting the $I/V$ relationships along the abscissa. Reversal potential (−40 mV) of the control experiment was taken as a reference.

This apparent difference is not due to the presence of PST, but is rather due to the lower driving force for cations, because the reversal potential in the top trace (Fig. 1*A*) was −37 mV and −27 mV in the middle trace (determined by linear regression, *see also* Fig. 3). (Reversal potentials of elementary currents are not 0 mV because of the cell-attached recording configurations. Therefore, reversal potentials of elementary current events can also be referred as membrane potentials (*see* Materials and Methods)). The apparently lower conductance for elementary currents in the bottom trace (Fig. 1*A*) is a true phenomenon, because the driving force for cations was higher than in the control conditions (membrane potential −48 mV).

The relationship between the elementary current amplitude and the presence of cholinesterase inhibitors is more clearly shown on Fig. 3, where elementary current was plotted *versus* pipette potential. Note that individual $I/V$ plots were shifted along the abscissa, and that the membrane potential of −40 mV was chosen arbitrarily to be the common reversal potential. Membrane potentials ranged from −20 to −65 mV, with an average of 40.3 ± 7.1 (mean ± SEM, $n = 7$), which is in agreement with previous reports (Lee, Miledi & Ruzzier, 1987; Ruzzier et al., *submitted*). From Fig. 3 it can be concluded that low doses of MSF (1.5 mM) and the presence of PST (20 $\mu$M) do not affect ACh-activated channel conuctance, which was 43.6 pS in control conditions and 46.3 and 44.5 pS in the presence of 20 $\mu$M PST and 1.5 mM MSF, respectively. These values are in agreement with conductance properties of the main (most frequently observed) conductance state reported elsewhere (Henderson et al., 1987; Ruzzier et al., *submitted*). However, at 5 mM MSF the channel conductance decreased to 29.7 pS. This effect was accompanied, as shown already (Fig. 2), by a reduction in elementary current dwell time. Therefore, the action of PST alters the channel kinetics, and the action of MSF results in altering the

channel kinetics and conductance. These results are consistent with the open-channel block of ACh-activated channels by cholinesterase inhibitors (Hille, 1984).

In the case of MSF the residency of the inhibitor molecule in the open channel seemed to be much shorter and may explain the (apparently) lower conductance of the channel. This may be due to the limited bandwidth of the recording apparatus. It may also be that the action of MSF may include another, as yet unidentified, mechanism which alters the channel conductance properties, but not via the blocking mechanism.

### MSF and Additional (Sub)conductance States

Interestingly, although no effect on elementary current dwell time was found at 1.5 mM MSF (*see above*), it appeared that at least two additional smaller conductance (subconductance) states were induced by MSF. This observation was studied further in two more patches.

It has been reported that in some cases ACh-activated channels in denervated muscle possess three conductance states, of which the highest and the lowest are very infrequent (open-channel probability less than 3% (Henderson et al., 1987); *see also* Fig. 4*B*). Figure 4 summarizes the conductance properties of these additional states. In this particular patch the occurrence of additional conductance states was around 9% of time spent in any elementary current state, with approximately half probability of occurrence in the high state (58.5 pS) and the low state (point conductances 23.5 and 24.1 pS). The major class of elementary currents had a conductance of 43.5 pS (Fig. 4*C*). The lowest conductance class was also observed infrequently as a substate (Fig. 4*A*).

As mentioned previously in the presence of a low concentration of MSF (1.5 mM) the kinetic properties of elementary currents did not change. But additional conductance classes of elementary current events were observed (Fig. 5). The major conductance class of 45.9 pS consisted of around 75% of time spent in any conductance state in this membrane patch (102-mV pipette potential). There was a higher conductance class of around 60 pS (*not shown*) whose probability of occurrence was less than 1% in these conditions. However, the presence of two additional conductance classes with smaller conductances was evident (*see* Fig. 5), with conductances of 36.2 and 19.4 pS. These events were seen as subconductance states (infrequent transitions between the most frequently observed conductance state to or from the smaller conductance level).

## Discussion

We have studied the effects of two cholinesterase inhibitors, prostigmine (PST) and methanesulfonyl fluoride (MSF) on ACh-activated elementary currents recorded in adult rat muscle fibers cultured for 5 to 10 days. The main finding of this work is that both agents (20 $\mu$M PST and 5 mM MSF) shorten the average dwell time of elementary current events which is consistent with the open-channel block mechanism (Hille, 1984).

Usually, elementary current events are grouped into bursts, which may be indicated by the longer time constant of the elementary current dwell-time distributions in control conditions (Fig. 2, left). This longer component seems to disappear in the presence of PST and MSF, which is again consistent with an open-channel block. If this is the mechanism, then one expects the intervals between the elementary current events (channel closed state) to be increased in the presence of cholinesterase inhibitors. In support of this we have found that the longer time constant of the distribution of intervals between the elementary current events (channel closed states were best fitted by at least two exponentials, *not shown*) was increased in the presence of PST and MSF in most patches. However, the data concerning intervals between the elementary currents should be treated with reservation, because the exact number of channels present in the patch was almost certainly more than two. The interpretation of such data has to be cautious, due to several experimental problems. For example, a large error is introduced by the probable overlap of signals from more than one channel in the patch, and there were at least two channels in our recordings. This problem is more severe in the presence of cholinesterase inhibitors, where elementary current dwell times are shorter and the intervals between them are larger. Bursts, interburst and intraburst intervals, as well as clusters of bursts, are under such conditions difficult to define. Therefore, we have not attempted to identify such categories (*see also* Clapham & Neher, 1984) and have restricted our interpretation only to the description of elementary current events, which undergo significant shortening in the presence of PST or MSF. As anticholinesterases were preset all the time, we cannot interpret our results in the light of a possible desensitization of ACh receptors. For this reason it would be interesting to look at the properties of ACh receptors after removal of PST or MSF.

Our second important finding is the additional conductance (subconductance) states at relatively low doses of MSF. The presence of subconductance states was described for cation-selective (e.g., Hamill & Sakmann, 1981; Barrett, Magleby & Pallotta,

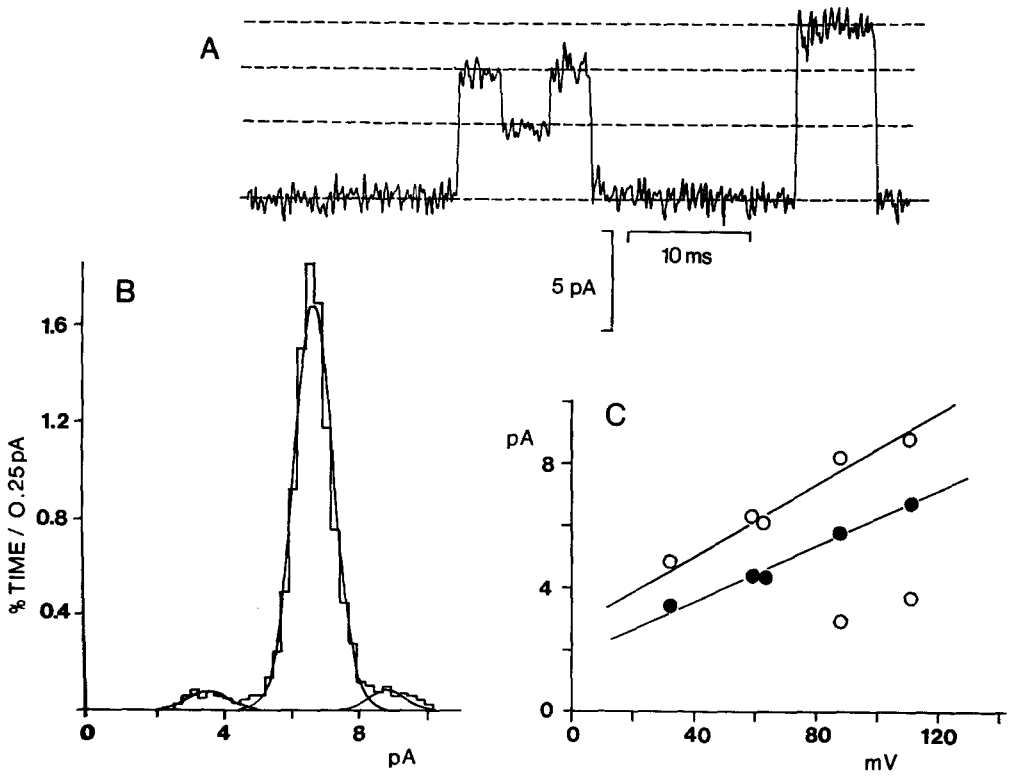


**Fig. 4.** Multiple conductance states of ACh-activated elementary currents in muscle fibers cultured for eight days. (*A*) Representative recording of elementary currents (111-mV pipette potential) showing three amplitude classes, the lowest being a substate (3 kHz, low-pass filtered). Dashed lines denote mean amplitudes determined from amplitude distributions in *B*. (*B*) Amplitude distribution of elementary current events. All points within elementary currents were plotted (three points were excluded during the transients of channel closing and opening). Three Gaussian curves were fitted to the distributions with the following parameters: 3.6 ± 0.6 pA (mean ± SD), 6.7 ± 0.6 pA and 8.8 ± 0.6 pA with relative areas under curves 3.9, 91.8, and 4.3%, respectively. (*C*) Relationship between amplitude of elementary current events and pipette potential. Lines were obtained by linear regression of the form: $y_5 = 0.0585 \cdot x_5 + 2.676$ (corr. = 0.99, $n = 5$), and $y_6 = 0.0435 \cdot x_6 + 1.850$ (corr. = 0.99, $n = 5$, filled circles denote the most frequent amplitude class). Point conductances for the low conductance states are 24.1 and 23.5 pS, assuming reversal potential of −43 mV (reversal potential equals membrane potential of the fiber; *see* Materials and Methods). Reversal potential was −42 mV for the lower slope line and −43 mV for the higher slope line.

1982; Hunter & Giebisch, 1987; Nagy, 1987) and anion-selective channels (e.g., Miller, 1982; Geletyuk & Kazachenko, 1985; Krouse, Schneider & Gage, 1986; Hughes et al., 1987; Smith, Zorec & McBurney, 1989). Their function is not clear. The presence of subconductance states certainly reflects the complex nature of conductive mechanisms as well as the complex structure of the channel. The presence of additional conductance states in the presence of MSF may represent partial blockade of the open channel. A similar observation was reported for calcium channels (Prod'hom, Pietrobon & Hess, 1987; Pietrobon, Prod'hom & Hess, 1988; *see also* Colquhoun, 1987). On the other hand, the existence of additional subconductance states may be due to an interaction of anticholinesterase drugs with the channel molecule away from the structures within the channel, but would interfere with the conductive properties of the channel. Whichever the mechanism, it is certainly interesting that a drug induces subconductance states, a phenomenon not reported previously. Similar effects of cholinesterase inhibitors can be expected with ACh-activated receptors in denervated noncultured muscle fibers, because their properties are almost identical to those recorded in long-term cultured muscle fibers (F. Grohovaz, P. Lorenzon, F. Ruzzier and R. Zorec, *submitted*).

In conclusion, it is worthwhile pointing out that the concentrations of PST needed to obtain shortening of ACh-activated channels are relatively low in comparison to the concentrations needed to obtain total inhibition of cholinesterase (Kordaš et al., 1975). Kordaš et al. showed that at 20 μM PST the inhibition of cholinesterase is about 50%, whereas at 1 mM MSF inhibition is 100%. Therefore, our

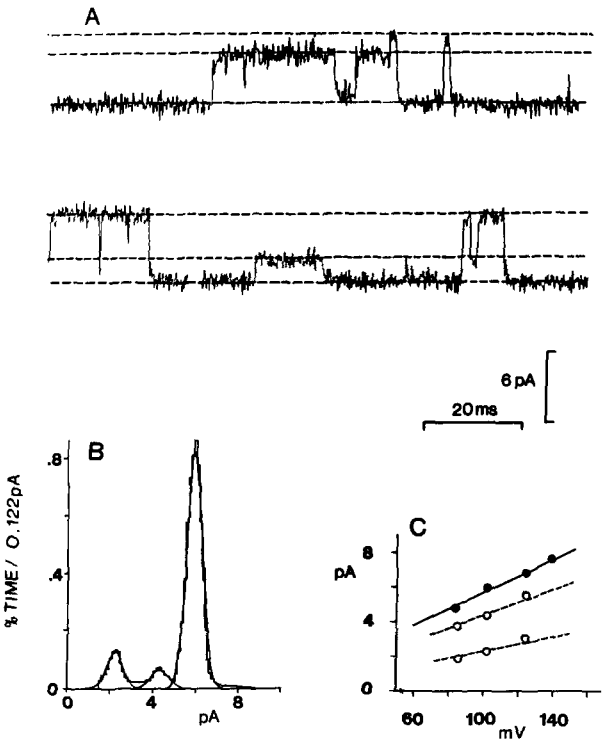


**Fig. 5.** Additional subconductance states of ACh-activated channels in the presence of 1.5 mM MSF. (*A*) Representative records of elementary current events in a cell-attached patch (pipette potential 102 mV, 3 kHz, low-pass filtered). Note the presence of transitions between various amplitude classes, which indicate the presence of subconductance states. Dashed lines denote average amplitudes determined from amplitude distributions in *B*. (*B*) Distribution of elementary current amplitudes obtained in the same cell-attached patch as in *A*. Three Gaussian distributions were fitted to the data with the following parameters: 2.2 ± 0.4 pA (mean ± SD), 4.3 ± 0.4 pA, and 4.9 ± 0.4 pA, with relative areas under the curves of 12, 7 and 81%, respectively. (*C*) Relationship between elementary current amplitude and pipette potential. Conductance of most frequently observed elementary current amplitude class (filled circles) was determined by linear regression to be 45.9 pS; regression equation $y_7 = 0.0459 \cdot x_7 + 1.071$, corr. = 0.99, $n = 4$, and reversal potential of −23 mV (which equals membrane potential; *see* Materials and Methods). Assuming this reversal for the smaller elementary current amplitude classes, conductances of 36.2 and 19.4 pS were obtained (parameters of lines are $y_8 = 0.0362 \cdot x_8 \cdot 0.775$, corr 0.99, $n = 4$, and $y_9 = 0.0194 \cdot x_9 + 0.370$, corr. = 0.98, $n = 4$).

results provide direct evidence for the advantage of MSF as a drug of choice in studies where the role of cholinesterase in the neuromuscular transmission is investigated.

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## References

Akaike, A., Ikeda, S.R., Brookes, N., Pascuzzo, G.J., Rickett, D.L., Albuquerque, E.X. 1984. The nature of the interactions of pyridostogmine with the nicotinic acetylcholine receptor-ionic channel complex. II. Patch-clamp studies. *Mol. Pharmacol.* **25**:102–112

Albuquerque, E.X., Aracava, Y., Cintra, W.M., Brossi, A., Schoenberger, B., Deshpande, S.S. 1988. Structure-activity relationship of reversible cholinesterase inhibitors: Activation, channel blockade and stereospecificity of the nicotinic acetylcholine receptor-ion channel complex. *Braz. J. Med. Biol. Res.* **21**:1173–1196

Barrett, J.N., Magleby, K.L., Pallotta, B.S. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* **331**:211–230

Bekoff, A., Betz, W.J. 1977. Physiological properties of dissociated muscle fibres obtained from innervated and denervated rat muscle. *J. Physiol.* **271**:25–40

Brehm, P., Henderson, L. 1988. Regulation of acetylcholine receptor channel function during development of skeletal muscle. *Dev. Biol.* **129**:1–11

Clapham, D., Neher, E. 1984. Trifluoperazine reduces inward ionic currents and secretion by separate mechanisms in bovine chromaffin cells. *J. Physiol.* **353**:541–564

Colquhoun, D. 1987. A new type of ion channel block. *Nature* **329**:204–205

Corey, D.P., Stevens, C.F. 1983. Science and technology of patch-recording electrodes. *In:* Single-Channel Recording. B. Sakmann and E. Neher, editors. pp. 53–68. Plenum, New York

Deana, A., Scuka, M. 1990. Time course of neostigmine action on the end-plate response. *Neurosci. Lett.* **118**:82–84

Del Castillo, J., Katz, B. 1957. A study of curare action with an electrical micro-method. *Proc. R. Soc. London B.* **146**:339–356

Dempster, J. 1989. Computer analysis of electrophysiological signals. *In:* Microcomputers in Physiology: A Practical Approach. pp. 53–93. IRL, Oxford

Eccles, J.C., MacFarlane, W.V. 1949. Actions of anti-cholinesterases on endplate potential of frog muscle. *J. Neurophysiol.* **12**:59–80

Geletyuk, V.I., Kazachenko, V.N. 1985. Single $Cl^-$ channels in molluscan neurones: Multiplicity of the conductance states. *J. Membrane Biol.* **86**:9–15

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100

Hamill, O.P., Sakmann, B. 1981. Multiple conductance states of single acetylcholine receptor channels in embryonic cells. *Nature* **194**:462–464

Henderson, L.P., Lechleiter, J.D., Brehm, P. 1987. Single channel properties of newly synthesized acetylcholine receptors following denervation of mammalian skeletal muscle. *J. Gen. Physiol.* **89**:999–1014

Henigman, F., Kordaš, M., Zorec, R. 1987. An inexpensive head stage for the 'patch-clamp' apparatus. *J. Physiol.* **391**:11P

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer, Sunderland (MA)

Hughes, D., McBurney, R.M., Smith, S.M., Zorec, R. 1987. Caesium ions activate chloride channels in rat cultured spinal cord neurones. *J. Physiol.* **392**:231–251

Hunter, M., Giebisch, G. 1987. Multi-barrelled K channels in renal tubules. *Nature* **327**:522–524

Katz, B., Miledi, R. 1972. The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* **224**:665–699

Katz, B., Miledi, R. 1977. Transmitter leakage from motor nerve endings. *Proc. R. Soc. London B* **196**:59–72

Kordaš, M. 1972*a*. An attempt at an analysis of the factors determining the time course of the end-plate current: I. The effects of prostigmine and of the ratio of $Mg^{2+}$ to $Ca^{2+}$. *J. Physiol.* **224**:317–332

Kordaš, M. 1972*b*. An attempt at an analysis of the factors determining the time course of the end-plate current: II. Temperature. *J. Physiol.* **224**:333–348

Kordaš, M., Brzin, M., Majcen, Z. 1975. A comparison of the effect of cholinesterase inhibitors on end-plate potential in the rat diaphragm. *Neuropharmacology* **14**:791–800

Krouse, M.E., Schneider, G.T., Gage, P.W. 1986. A large anion-selective channel has seven conductance levels. *Nature* **319**:58–60

Kuba, K., Albuquerque, E.X., Daly, J., Barnard, E.A. 1974. A study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate, on time course of end-plate currents in frog sartorius muscle. *J. Pharmacol. Exp. Ther.* **189**:499–512

Kuba, K., Tomita, T. 1971. The effect of prostigmine on the time-course of the end-plate potential in the rat diaphragm. *J. Physiol.* **213**:533–544

Lamb, T. 1985. An inexpensive digital tape recorder suitable for neurophysiological signals. *J. Neurosci. Methods* **15**:1–13

Lee, S., Miledi, R., Ruzzier, F. 1987. The development of tetrodotoxin-resistant action potentials in long-term organ culture of rat muscle. *Q. J. Exp. Physiol.* **72**:601–608

Magleby, K.L., Stevens, C.F. 1972*a*. A quantitative description of end-plate currents. *J. Physiol.* **223**:173–197

Magleby, K.L., Stevens, C.F. 1972*b*. The effect of voltage on the time course of end-plate currents. *J. Physiol.* **223**:151–171

Miller, C. 1982. Open-state substructure of single chloride channels from *Torpedo* electroplax. *Phil. Trans. R. Soc. London. B* **299**:401–411

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C., Sakmann, B. 1986. Molecular distinction between fetal and adult forms of muscle acetylcholine receptors. *Nature* **321**:406–411

Nagy, K. 1987. Subconductance states of single sodium channels modified by chloramine-T and sea anemone toxin in neuroblastoma cells. *Eur. Biophys. J.* **15**:129–132

Pascuzzo, G.J., Akaike, A., Maleque, M.A., Shaw, K., Aronstam, R.S., Rickett, D.L., Albuquerque, E.X. 1984. The nature of the interactions of pyridostygmine with the nicotinic acetylcholine receptor-ionic channel complex: I. Agonist, desensitizing and binding properties. *Mol. Pharmacol.* **25**:92–101

Pietrobon, D., Prod'hom, B., Hess, P. 1988. Conformational changes associated with ion permeation in L-type calcium channels. *Nature* **333**:373–376

Prod'hom, B., Pietrobon, D., Hess, P. 1987. Direct measurement of proton transfer rates to a group controlling the dihydropyridine-sensitive Ca channel. *Nature* **329**:243–246

Ruzzier, F., Zorec, R. 1988. Acetylcholine-activated channels in cultured adult rat muscle fibres acquire denervation-like properties. *In:* Abstracts of the 11th Annual Meeting of the European Neuroscience Association, Zurich, 4–8 Sept., 17.2

Schuetze, S.M., Role, L.W. 1987. Developmental regulation of nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* **10**:403–457

Scuka, M. 1987. The effect of anticholinesterase drugs on the ionophoretically-evoked end-plate currents. *Pfluegers Arch.* **410**:428–432

Scuka, M. 1989. The response of end-plate receptors to single transmitter quanta during anticholinesterase treatment. *In:* Neuromuscular Junction. L.C. Sellin, R. Libelius, and S. Thesleff, editors. p. 614. Elsevier, Amsterdam

Shaw, K.-P., Aracava, Y., Akaike, A., Daly, J., 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. *Mol. Pharmacol.* **28**:527–538

Smith, S.M., Zorec, R., McBurney, R.N. 1989. Conductance states activated by glycine and GABA in rat cultured spinal neurones. *J. Membrane Biol.* **108**:45–52

Steinbach, J.H. 1989. Structural and functional diversity in vertebrate muscle nicotinic acetylcholine receptors. *Annu. Rev. Physiol.* **51**:353–365

Zorec, R., Henigman, F., Mason, W.T., Kordaš, M. 1991. Electrophysiological study of hormone secretion by single adenohypophyseal cells. *Methods Neurosci.* **4**:194–210

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