Meproadifen enhances activation and desensitization of the acetylcholine receptor-ionic channel complex (AChR): single channel studies

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The effects of the quaternary agent meproadifen on ACh-activated channel currents were studied on myoballs cultured from hind limb muscles of neonatal rats. Meproadifen (0.02–0.1 μM) combined with ACh (0.1–0.3 μM) in the patch pipette caused an increase, followed by a decrease, in the frequency of channel openings. At concentrations greater than 0.2 μM the initial phase was not detected and a rapid and marked reduction in the opening frequency was observed. Meproadifen (up to 2.5 μM) produced no change in the duration or conductance of the open state of ACh-activated channels. In addition, this agent induced the appearance of events with a marked increase in the 'noise' during the opening phase. The lack of effect under inside-out patch conditions suggested that meproadifen binds to a site located at the external portion of the nicotinic macromolecule and has no access to it through the cell membrane. This study indicated that non-competitive antagonists such as meproadifen can facilitate receptor activation and desensitization.

Meproadifen *Nicotinic acetylcholine receptor* *Single channel current* *Endplate region* *Activation-desensitization*

1. INTRODUCTION

Previous studies on the acetylcholine receptor-ionic channel complex (AChR) of the frog neuromuscular junction and the *Torpedo* electric organ membranes have shown that the quaternary agent meproadifen (fig.1) increases the affinity of the agonist for its binding site and enhances desensitization [1,2]. The electrophysiological assertions were based on a series of observations including: (i) voltage- and time-dependent depression of the endplate current (EPC) peak amplitude as revealed by nonlinearity and hysteresis in the current-voltage relationship; (ii) rundown of the EPC amplitude with repetitive nerve stimulation, and (iii) reduction of junctional and extrajunctional response to microiontophoretic application of ACh. Moreover, persistence of these effects in the absence of any significant change in either the time constant of the EPC decay phase (τ_{EPC}) or the single channel conductance and mean lifetime determined from noise analysis indicated that meproadifen exerts its affects on activation of ionic channels without affecting the conducting species of the AChR complex.

Our objectives are to observe the effects of meproadifen on properties of single currents; to

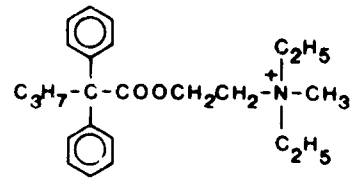

Fig.1. Chemical structure of meproadifen.

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characterize at the level of the elementary currents enhancement of desensitization induced by an increase in agonist affinity for its receptor site; to estimate the location of the meproadifen binding site on the AChR complex and to determine the access route to it by a selective application of the drug to either side of the cell membrane under different patch-clamp conditions. An abstract of this work has been presented [3].

2. MATERIALS AND METHODS

The single channel current recordings were performed at 10°C on myoballs cultured from hind limb muscles of 1–2-day-old rat pups using the improved patch-clamp technique [4]. Myoballs, which formed spontaneously (i.e., without addition of colchicine) in 1–2-week-old cultures, were used. Upon removal of cultures from the incubator, the nutrient medium was replaced with Hanks' solution [composition (mM): 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; Hepes, 10; pH 7.2] to which was added tetrodotoxin (TTX, 0.3 μM) to abolish the cell contraction. An LM-EPC-5-Patch System (List Electronic) was used to record single channel currents which were filtered to 3 KHz (second order, Bessel low pass) and stored on FM magnetic tape for computer analysis. The data were sent to a PDP 11/40 minicomputer through a fourth-order Butterworth (low-pass) filter (1–3 kHz), to improve the signal-to-noise

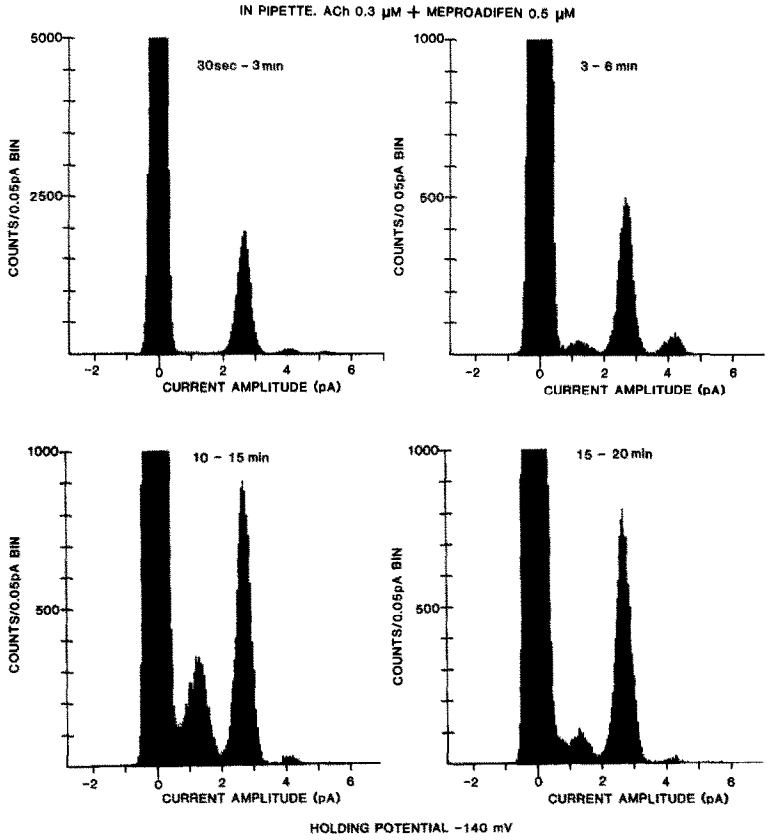

Fig.2. Total amplitude histograms of channel currents recorded at various time intervals (pipette contained a mixture of 0.3 μM ACh and 0.5 μM meproadifen). The abscissa shows the current amplitude in pA. The largest peak, centered around 0 mV, represents the noise level of the non-conducting state of AChR complex. The position of the second largest peak on the abscissa (2.7 pA) indicates the amplitude of the single channel current. The peak at amplitude of 4.2 pA corresponds to ACh-activated channels with higher conductance frequently observed in rat myoballs under control conditions [8].

ratio and digitized at 2–10 KHz. An automated analysis provided amplitude and open time histograms from which the conductance and mean open time of ACh-activated channels were estimated. Details of the patch-clamp experiments and the cell culture procedure have been described [5].

3. RESULTS

Interaction of meproadifen and acetylcholine at the nicotinic receptor-ion channel complex of neonatal rat myoballs: In our recordings, a high percentage of ACh-channel openings disclosed a conductance value (obtained from the slope of the current-voltage plot) of 20 pS. Events with conductance of 33 and 10 pS were also observed [6]. Application of meproadifen (0.02–2.5 μM) together with ACh (0.3 μM) to the external surface of the cell membrane via the patch micropipettes did not alter the properties of ACh-activated single channel currents, i e., both channel conductance and the duration of the open state were similar to control values obtained with ACh alone inside the micropipette (fig.2,3). The open channel histograms showed an excessive number of short events which contributed to a departure from a single exponential distribution similar to that seen under control conditions. However, meproadifen caused a marked effect on the frequency of ACh-activated channel openings (fig.4, table 1). Meproadifen (>0.2 μM) produced an immediate and a concentration-dependent decrease of the opening frequency, detectable at the first minute after the establishment of the gigaohm seal, such that at above 2.5 μM no channel activity could be recorded. An initial increase in the frequency of

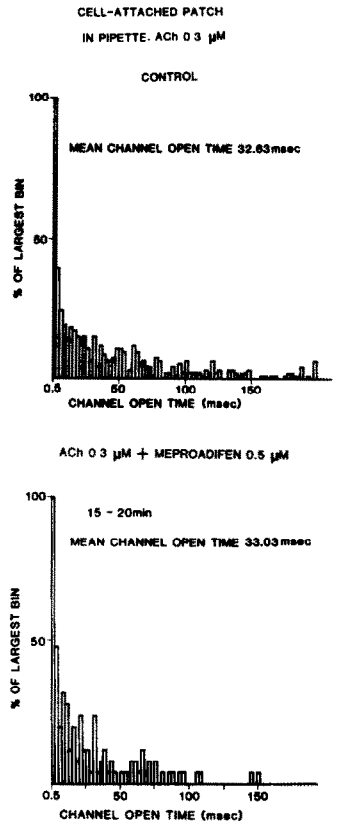

Fig.3. Open time histograms of channel currents recorded with the patch micropipettes containing ACh (0.3 μM) alone and together with meproadifen (0.5 μM). Holding potential −140 mV.

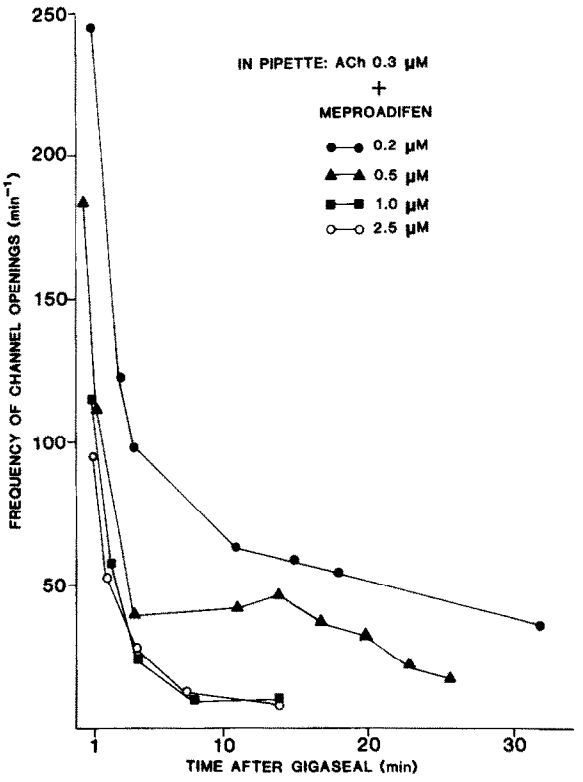

Fig.4. Concentration-dependent effect of meproadifen on the frequency of channel openings. Gigaohm seals were established with the pipette containing ACh 0.3 μM and meproadifen at different concentrations.

Table 1

Effect of meproadifen on frequency of ACh-activated channel openings[a]

Condition of drug application		Meproadifen concentration (μM)	Frequency of channel openings (min^{-1})		
			Control	1 min	15 min
I.	Bathing superfusion				
	A. Cell-attached patch	10 μM	293	–	292
	B. Inside-out patch	5 μM	197	–	193
II.	Micropipette: 0.3 μM ACh + meproadifen	0.2 μM	–	246	58
		0.5 μM	–	153	36
		1.0 μM	–	115	10

[a] The values refer to the frequency (number of events per min) determined under control condition and 15 min after starting drug superfusion in IA and B and 1 and 15 min after establishment of the gigaohm seals in II. In IA and B the concentration of ACh in the micropipette was 0.3 μM

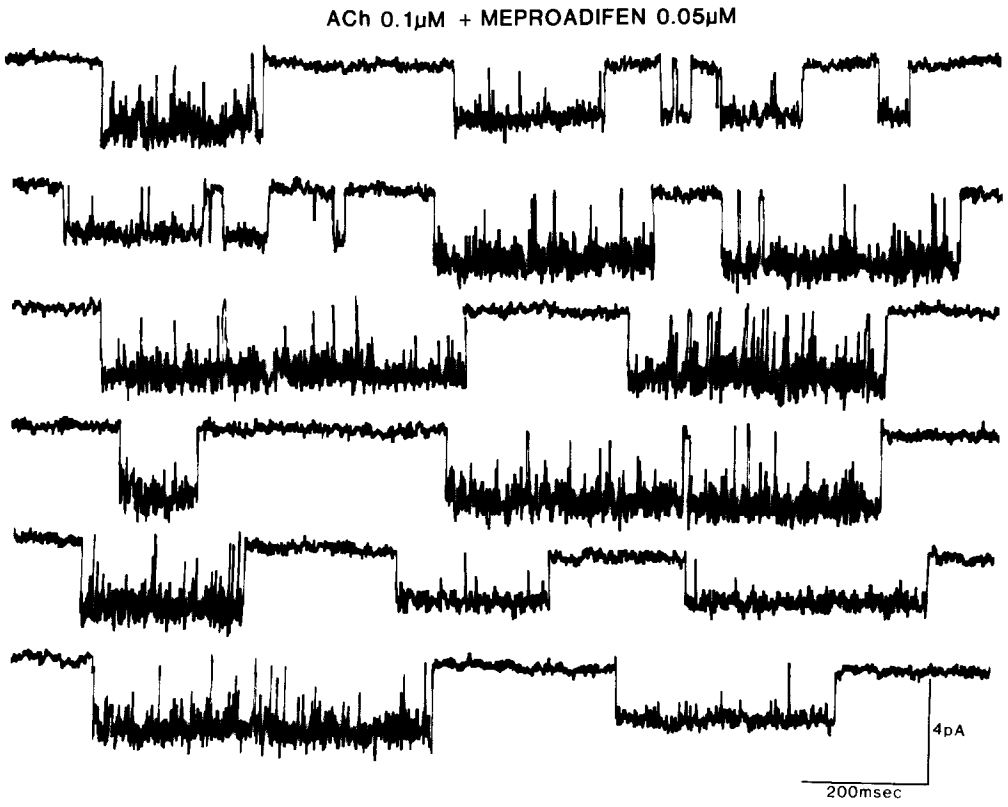

Fig. 5. Samples of ACh-activated channel currents recorded from cell-attached patches in the presence of meproadifen. Note in addition to the unchanged channel currents, the presence of many events with enlarged baseline and flickers during the open phase.

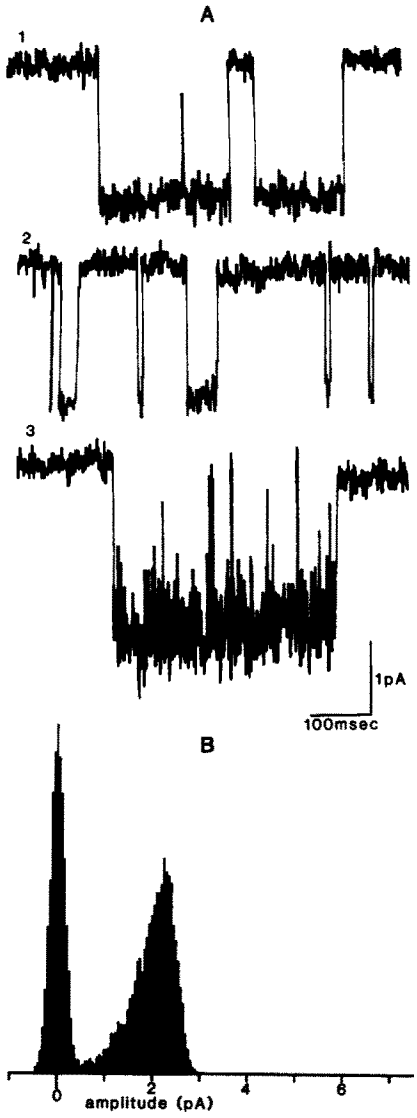

Fig.6. (A) Baseline broadening during the open state of ACh-activated channel openings induced in the presence of meproadifen. Patch micropipettes contained (1) ACh, 0.2 μM; (2) ACh, 0.2 μM; bupivacaine, 20 μM; (3) ACh, 0.1 μM; meproadifen, 0.05 μM. Holding potential −100 mV. In contrast to meproadifen, notice the short events with normal thickness of the baseline in the presence of bupivacaine (2). (B) Amplitude histogram of the events shown in (3). Bandwidth 3 kHz.

channel openings, followed by a decrease, was observed at below 0.1 μM. Grouping of events with multiple simultaneous openings characterizing the initial phase gradually disappeared as the effect of the drug proceeded and bursts between long silent intervals were seen. We also observed the appearance of events with low conductance (~10 pS) after 3 min exposure to 0.5 μM meproadifen together with 0.3 μM ACh (fig.2,4). These events, which became clearly noticeable during the following 10 min, gradually disappeared after 15 min exposure to the drug. Moreover, in the presence of meproadifen (0.05 μM), some channel openings activated by ACh (0.2 μM) exhibited a marked thickening of the base line during the opening phase which appears to be composed of high frequency events (fig.5). Since the distribution of amplitude points is skewed to the left (fig.6B), it is likely that the apparent increase in current fluctuations is due to the presence of inadequately detected short closures. At high concentration of meproadifen (>0.2 μM) these events were rarely observed. Fig.6A shows this phenomenon in more detail and also provides a comparison of these events with single channel currents activated by ACh alone and by ACh in the presence of a typical open channel blocker, bupivacaine. Neither ACh alone nor in combination with bupivacaine exhibited the perturbations of the opening state observed with meproadifen.

Superfusion of bathing solution containing a high concentration of meproadifen (5–10 μM) alone to the external face in cell-attached patch or to the cytoplasmic side of the cell membrane in inside-out patch produced no effect on the amplitude and duration of channel currents activated by ACh (fig.7), as in the case of application via the pipette. However, the changes in frequency of events observed with ACh plus meproadifen in the pipette and the widening of the baseline during the open channel were not present with any of the other methods of application (table 1).

4. DISCUSSION

This study has revealed actions of meproadifen on ACh-activated single channel currents. In a previous study, it was shown that the agent enhances the agonist-mediated conversion of the nicotinic receptor from a state of low affinity to one of high affinity which is associated with a slow decline of the permeability response of the muscle membrane [1,2]. In agreement with the voltage-clamp data which disclosed no change in the decay

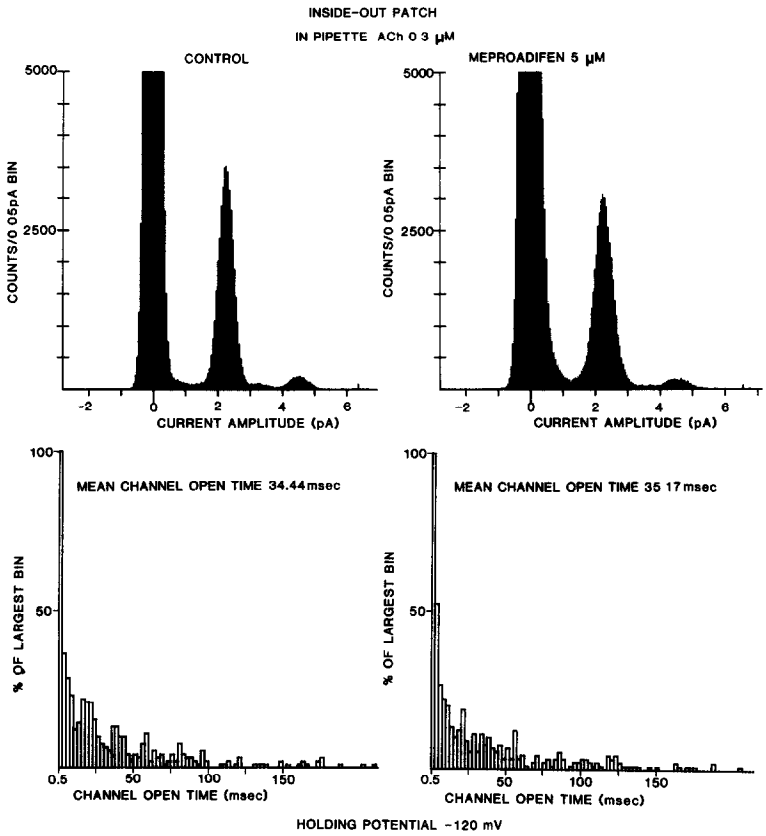

Fig.7. Application of meproadifen via the bath in inside-out patch condition after the establishment of the gigaohm seal. Total amplitude and open time histograms correspond to single channel currents activated by 0.3 μM ACh before (left) and after 15 min exposure to 5 μM meproadifen. The peaks located at 2.25 and 4.5 pA on the abscissa represent respectively the current amplitude of a single channel opening and two channels opening simultaneously.

time constant of the endpoint currents (EPC), single channel current recordings revealed that meproadifen did not significantly change the single channel open time. However, ACh-activated single channels recorded in the presence of very low concentrations of meproadifen (0.02–0.01 μM) produced an initial increase in the frequency of channel-opening events which was followed by a significant decrease in channel activation. The initial increase in channel opening frequency was not detected at concentrations of the agent greater than 0.2 μM, and only the latter phase of the drug action, i.e., a rapid and marked depression of channel opening frequency was observed (fig.4, table 1). Enhanced grouping of channel openings followed by long quiescent periods may reflect enhancement of the receptor desensitization induced by meproadifen [7]. On the other hand, at the macroscopic level, this phenomenon might be evidenced by a use-dependent depression, or run-down, of the EPC amplitude and the marked depression of the junctional and extrajunctional sensitivity to microiontophoresis of ACh observed in the presence of 5–20 μM meproadifen [2]. Moreover, at suitable concentrations this agent induced appearance of events with an increased number of very short closures and broadening of the baseline during the open state. Although further studies are required to evaluate this alteration of the baseline during channel opening, it is tempting to speculate whether these altered events are correlated to the high affinity species of the nicotinic AChR complex. A similar effect, but to a lesser extent, was exhibited by other agents such as pyridostigmine

which aside from anticholinesterasic activity also increased the affinity of the agonist for its binding site, enhanced receptor desensitization and behaved as a weak agonist at the AChR complex [5]. This pattern was not shared by agents such as bupivacaine (see fig.6) which acted essentially as an open channel blocker and had no effect on agonist-induced receptor desensitization [6,8]. The absence of any alteration of the properties (amplitude and channel lifetime) of the single channel currents activated by ACh, suggested that meproadifen did not affect the conducting species of AChR complex. As proposed for phenothiazine neuroleptics [9], which cause an increase in the affinity of ACh for its binding site and induce desensitization, meproadifen at suitable concentrations may interact with the resting, nonconducting species of the AChR complex to stabilize it or shift the equilibrium between the concentrations of the closed, activated and open AChR in favor of a desensitized conformation. However, meproadifen and phenothiazines may bind to a different class of sites, proposed for drugs which stabilize the high affinity species of the AChR complex, which includes one which is sensitive and one insensitive to voltage [10–12]. In contrast to the phenothiazines, the enhancement of desensitization by meproadifen may be due to its preferential binding to a voltage-sensitive site at the AChR complex. In addition, the appearance of low-conductance events (fig.2) in the presence of this agent could be related to a weak agonistic property similar to that of pyridostigmine [5]. However, meproadifen at concentration range of 0.1–1 μM did not show such effect. On the other hand, since the low-conductance events can be activated, albeit infrequently, by ACh itself [6], it is more probable that the frequency of these events is increased as a consequence of facilitation of receptor activation by meproadifen.

One important aspect of the interactions of meproadifen with the AChR complex which can be determined using patch-clamp technique is the location of the binding site(s) responsible for its effects. The selective application of meproadifen to the intracellular side of the cell membrane under inside-out patch condition, in contrast to application of the drug together with the agonist to the extracellular face of the membrane, did not affect the kinetics of the ACh channel activation. Similarly, when meproadifen was superfused in the bathing medium outside the pipette in cell-attached patch condition no significant change was observed in the properties of the single channel currents. Assuming that meproadifen is not able to pass through the space between the micropipette and the cell surface after the establishment of the gigaohm seal [4,5], these results suggest that the drug also does not diffuse through the cell membrane. Similar findings were reported for other quaternary agents such as bupivacaine methiodide [6] and QX-222 [13]; however, the anticholinesterasic agent pyridostigmine has a definitive effect on the ACh channel activation under any patch-clamp conditions [5]. Additionally, the absence of any effect when this agent was applied to the bathing medium under inside-out condition, suggests that most likely there is no site for meproadifen interactions at the internal segments of the AChR macromolecule. The availability of more selective agents such as bupivacaine, which exhibits a powerful blockade of the open conformation of the ACh channels, and drugs such as meproadifen and phenothiazines, which have the capability of accelerating the desensitization of the nicotinic receptor, has allowed a distinction between the different sites at the ionic channel of AChR complex which are known to bind a great variety of noncompetitive antagonists [14].

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