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The Mean Conductance and Open-Time of the Acetylcholine Receptor Channels Can Be Independently Modified by Some Anesthetic and Convulsant Ethers

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SUMMARY

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The effects of two anesthetic ethers, methoxyflurane and diethyl ether (ether), and one convulsant ether, fluothyl, were examined on miniature end-plate currents and on end-plate current fluctuations at the frog neuromuscular junction. Either methoxyflurane or fluothyl was found to reduce the amplitude and the half-decay time of the miniature end-plate currents. When the concentration of methoxyflurane was raised from 10^{-4} to 5.10^{-4} M its effect on the miniature end-plate current amplitude was increased but that on the half-decay time remained unaltered. The effect of ether on the miniature end-plate current was dose-dependent: at 10^{-3} M it reduced the half-decay time without affecting the amplitude, whereas at 10^{-2} M it increased the half-decay time and decreased the amplitude. Analysis of end-plate current fluctuations revealed that the effects of methoxyflurane (5.10^{-4} M) and ether (10^{-2} M) on the mean conductance and open-time of the acetylcholine receptor channels were comparable to their effects on the amplitude and the half-decay time of the miniature end-plate current. It is concluded that the effect of such structurally "non-specific" drugs comprises a combination of discrete and distinguishable events which are specific and dose-dependent for each of the agents used.

INTRODUCTION

In a previous publication (1) we showed that some volatile anesthetic and convulsant ethers shared common effects on synaptic transmission at the frog neuromuscular junction: at low doses, agents of either type enhanced release of transmitter, whereas at higher doses they decreased post-synaptic sensitivity. The convulsants elicited effects of the same quality as the anesthetics, but they also did so at relatively higher concentrations than the anesthetics. We suggested that these ethers could dissolve in specific subregions of the membrane, in a manner analogous to the formation of a regular solution. Conversely, these subregions, acting as solutes, selected ethers of comparable solubility parameter (i.e., anesthetics) over those with a less favorable solubility parameter (i.e., convulsants). This view implied an element of specificity in the pharmacodynamic activity of these so-called "non-specific" agents. Verification of this hypothesis necessitated, among other things, a study of the effect of these agents at the level of the single molecular event elicited by ACh[1] at the end

[1] The abbreviations used are: ACh, acetylcholine; MEPC, miniature end-plate current; $t_{1/2}$, half-decay time; MEPP, miniature end-plate potential.

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plate. Of major concern here are two parameters which may or may not be interrelated: mean open-time of the Ach-receptor channel (τ) and single channel conductance (γ). These parameters could be assessed independently from an analysis of miniature end-plate currents (MEPC) and from ACh-induced end-plate current fluctuations (ACh noise), as measured in an isolated preparation that had been subjected to the effect of the relevant drugs. The results of the present study support our contention that the effect of structurally "non-specific" drugs comprises a combination of discrete and distinguishable molecular events that are specific for each of the agents used.

MATERIALS AND METHODS

Preparation. Experiments were performed on neuromuscular junctions of the m. tibialis anterior longus of the frog (Rana ridibunda). The muscle was dissected from the animal and pared down to a layer 1–3 fibers thick. Nerve-muscle junctions were readily observable under Nomarski optics (Zeiss). The fibers in this muscle have the same length as those of m. cutaneous pectoris, but have double the diameter, which improves conditions for voltage clamping and space uniformity.

Solutions and drugs. The basic Ringer's solution had the following composition (mM): NaCl, 116; KCl, 2.5; $CaCl_2$, 1.8; and Tris maleate, 2. The solution was adjusted with HCl or NaOH to pH 7.4 ± 0.1. The drugs used were: methoxyflurane (Penthrane-Abbott), fluothyl or hexafluorodiethyl-ether (known also as Indoklon-Ohio) synthesized by Dr. A. Goldschmid (2) and diethylether (ether—Abbott). These were dissolved in Ringer's solution within one hour of the experiment, and kept in closed bottles to prevent evaporation. The hypertonic solution used for the detubulation of the muscle fibers contained 1 M ethylene glycol added to the basic Ringer solution.

Bath applications. The preparation was placed in a 3 ml tissue bath. In the MEPC experiments the test solution was washed in and out for 5 min at a flow rate of 20 ml/min. The preparation was kept at room temperature, 20–23°. In the Ach-noise experiments, a constant flow of cooled bathing solution at a rate of 20 ml/min kept the preparation at a given temperature in the range of 7–10°, but the temperature within one experiment varied within ±0.5° only. Again, wash-in or wash-out periods of 5 min were allowed.

Recording of MEPCs. The MEPC is the current that flows through the end-plate membrane in response to a single quantum of ACh. Synapses were located visually with Nomarski optics (3), and two glass micro-pipettes filled with 2 M KCl (tip resistance: 1–5 MΩ) were inserted into the muscle cell in the synaptic region. One microelectrode measured the membrane voltage while the other was employed to inject current into the cell. The membrane voltage was clamped (usually to −70 mV) with a feed-back amplifier similar to that of Anderson and Stevens (4). The voltage signal was led through a P-1 preamplifier (Bioelectric) to a Tektronix oscilloscope (5103), and to the feed-back amplifier. The current signal from the feed-back amplifier was led through a steep roll-off filter (Krohn-Hite 335) and displayed on the screen of a storage oscilloscope (Tektronix 5103). For the measurement of the time-course of MEPCs the signal was filtered through a bandwidth of 0–3000 Hz and displayed at a fast sweep rate. For amplitude measurements, the filter bandwidth was 0–1000 Hz, and the sweep rate was slow. The oscilloscope display was photographed on polaroid film and further analyzed. To measure the time of MEPC decay, a line was drawn at half the amplitude and the time of decay of the MEPC to half its peak amplitude was determined. The $t_{1/2}$ were averaged for 3 to 5 different MEPC records and the test value expressed as percent of control. The coefficient of variation of $t_{1/2}$ in each group of MEPCs was usually less than 10%. Also, in each individual experiment the change in time constant was always statistically significant: ($p < 0.005$; t-test). In a number of cases, the MEPCs were photographically enlarged, a line was drawn at half the amplitude and the amplitude values were plotted on semilogarithmic coordinates. A straight line through the points was fitted by the least squares method to values between 80–20% of the MEPC amplitude to give the time

constant of decrease. To determine the amplitude of MEPCs, at least 85 signals were averaged. Experiments were accepted only if drug effect reversed to within 10% of the control level after the end of the wash-out period. Usually, reversibility was better than ±10%, being ±5% for the amplitude of the MEPCs and ±1% for the half-decay time. The ACh noise is an expression of the random opening and closing of the ACh-induced ionic channels and can lead, after suitable analysis to an estimation of the single channel parameters, γ and τ, as defined in the following section.

ACh noise measurement. The same electrodes and voltage clamp amplifier were used as in the MEPC experiments. A third, high resistance micropipette (tip resistance 20–50 MΩ), filled with 2 M ACh was positioned 50 μ over the clamped synapse, and ACh was iontophoretically applied by reversing the direction of the holding current (2–10 nA) for 20 sec or less. The synaptic current signal was led from the voltage clamp amplifier into two separate channels of an analogue tape-recorder (Hewlett-Packard magnetic recording system—3955). One channel was D.C.-coupled and recorded the average synaptic current (μ_I) at low amplification, whereas the other channel was A.C.-coupled (Bandwidth: 1–500 Hz) and recorded synaptic current fluctuations (ACh-noise) at a high gain. The Ach-noise trace was sampled with a varian 620-L minicomputer, and the digitized noise data were stored on a digital tape. Finally the digital tape was analyzed on a CDC-3600 computer. The sampling rate was 615 Hz and for each ACh-noise epoch 5000 data points were acquired. From these data, 512 spectral points were calculated in the frequency range of 1 to 307 Hz, after subtraction of the background noise. The mean open-time of the channel (τ) was calculated as $\tau = 1/2\pi f_c$ where f_c was the corner frequency of a Lorentzian function fitted to the spectral points by the computer (least-squares fit). The Lorenzian function had the following form: $S(f) = S(0)/(1 + f^2/f_c^2)$, where S is the power density as a function of the frequency, f is the frequency and f_c the corner frequency. The single channel conductance (γ) was calculated as: $\gamma = S(0)/[2\mu_I \cdot \tau \cdot (V - V_{eq})]$ where $S(0)$ is the power density at a frequency of 0, V the holding potential, V_{eq} the reversal potential, μ_I the average synaptic current (measured separately) and τ the channel open-time (4).

For each cell, three different spectra were calculated: control, test (with the drug), after washout. Results were considered valid only if both τ and γ reversed to within 10% of their control value after washout.

Determination of the reversal potential. Cells were detubulated with a hypertonic solution of ethylene-glycol to prevent muscle contraction. Synapse localization, voltage clamping and ACh application were the same as described above, except that the iontophoretic current pulses had an amplitude of 50 nA and a duration of 2 sec and were delivered every 30 sec. The holding potential was varied between −60 mV and +40 mV, and the clamping currents were recorded on a storage oscilloscope, photographed and analyzed. A typical recording from such an experiment is shown in Fig. 1.

RESULTS

Fluorinated ethers accelerate the rate of decay of the MEPCs and reduce their amplitude. In a previous study (1) we found that fluorinated ethers decrease the amplitude of MEPPs. In the present study the drugs were used at concentrations where the MEPP amplitude would be reduced by half: 10^{-4} M for methoxyflurane and 10^{-3} M for fluothyl. The effect of 10^{-4} M methoxyflurane can be seen in Fig. 2. It is clear that the decay of the MEPCs in its presence is faster (MEPCs on the fast time base) and that their amplitude is smaller (MEPCs on the slow time-base). When the MEPCs shown in Fig. 2 were photographically enlarged and drawn on semilogarithmic coordinates (see METHODS), the time course with methoxyflurane remained exponential, with no evidence of a slower second phase as was found with local anesthetics (5–8), and some general anesthetics (9). In five different experiments with 10^{-4} methoxyflurane, the half decay time of MEPCs decreased by 31% ± 2 (SEM)[2] and the amplitude decreased by 21% ± 1. Similar results were obtained with fluothyl (10^{-3}

[2] Error values throughout the paper are SEM.

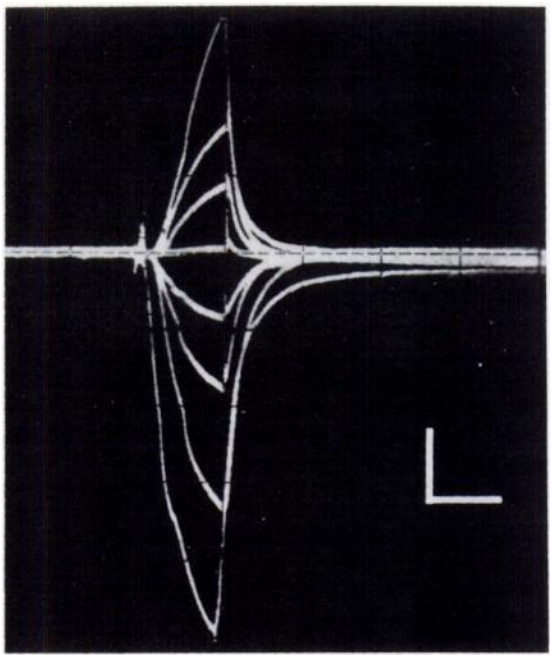

FIG. 1. *Reversal potential experiment (in methoxyflurane $5 \cdot 10^{-4}$ M)*

The membrane potential was clamped (from the lower trace to the upper) at: −60, −40, −20, −10, 0, +10, +20 and +40 mV. The photograph is a superposition of the 8 traces of clamping current. ACh was applied by iontophoretic pulses of 50 nA and 2 sec duration. The calibrations are 100 nA and 2 sec. Reversal potential in this case was estimated as −2 mV.

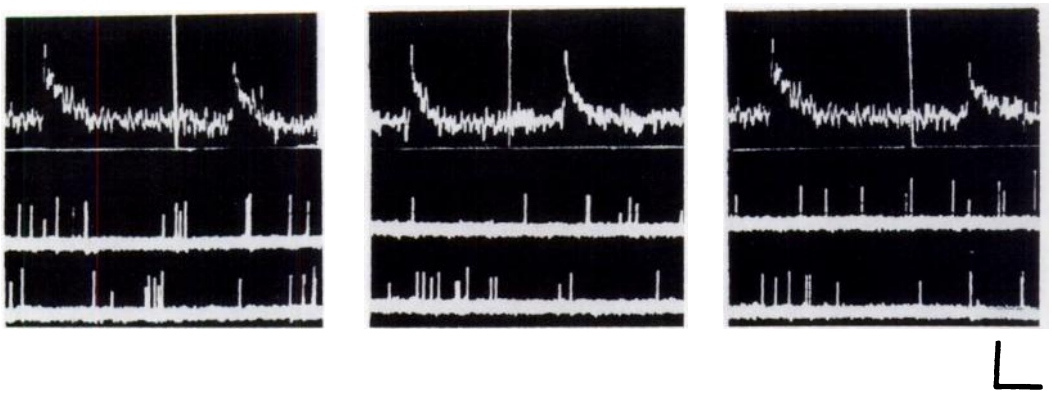

FIG. 2. *The effect of methoxyflurane (10^{-4}M) on the MEPCs*

The three sets of photographs are (left to right): control, methoxyflurane and control after washout, and were taken in the same cell. Upper traces are at a fast sweep rate, showing the effect on the time of decay. The two lower traces are at slow sweep and show the effect on the amplitude. In this case the half-decay time was shortened by 30% and the amplitude decreased by 25%. Calibrations are 2.5 nA and 5 msec for the upper and 5 nA and 1 sec for the lower traces.

M), the half-decay time decreasing by 33% ± 0.5 (6 experiments), and the amplitude decreased by 32% ± 1 (6 experiments). All these differences were significant at the 0.1% level (*t*-test). This finding strengthens our conclusion from previous work (1) that both drugs share a common mechanism of action on the muscle end-plate.

To study the dose dependence of these phenomena we also examined the effects of methoxyflurane at a higher concentration, $5 \cdot 10^{-4}$ M. Whereas the half-decay time of the MEPC was not further decreased [$\Delta t_{1/2} = -31\% \pm 1$ (5 experiments)], there occurred a further decrease in the MEPC amplitude [Δamplitude $= -43\% \pm 2$ (5 experiments)]. The difference between the effects of the drug at 10^{-4} M and $5 \cdot 10^{-4}$ M on the amplitude of the MEPC was significant at the 0.1% level (*t*-test). Thus, it appears that the effects of methoxyflurane on the time course of the MEPC can be separated from its effects on the MEPC amplitude.

Ether has a dual effect on the rate of decay of the MEPC and reduces its amplitude. As already reported by Gage and Hamill (9), ether at a relatively low concentration (10^{-3} M) shortened the duration of the MEPC (Fig. 3*a*). But, when the concentration was increased to 10^{-2} M the duration of the MEPC was increased (Fig. 3*b*). When the MEPC traces were enlarged and plotted on semi-logarithmic coordinates their decay was again exponential, with no evidence for a second, slow phase of decline. At 10^{-3} M ether had no effect the MEPC amplitude, but at 10^{-2} M reduced the amplitude by 45% ± 1 (5 experiments).

The MEPC reversal potential is unaffected by the drugs. To examine whether drug effect on the MEPC amplitude was mediated through a change in reversal potential, we measured the reversal potential

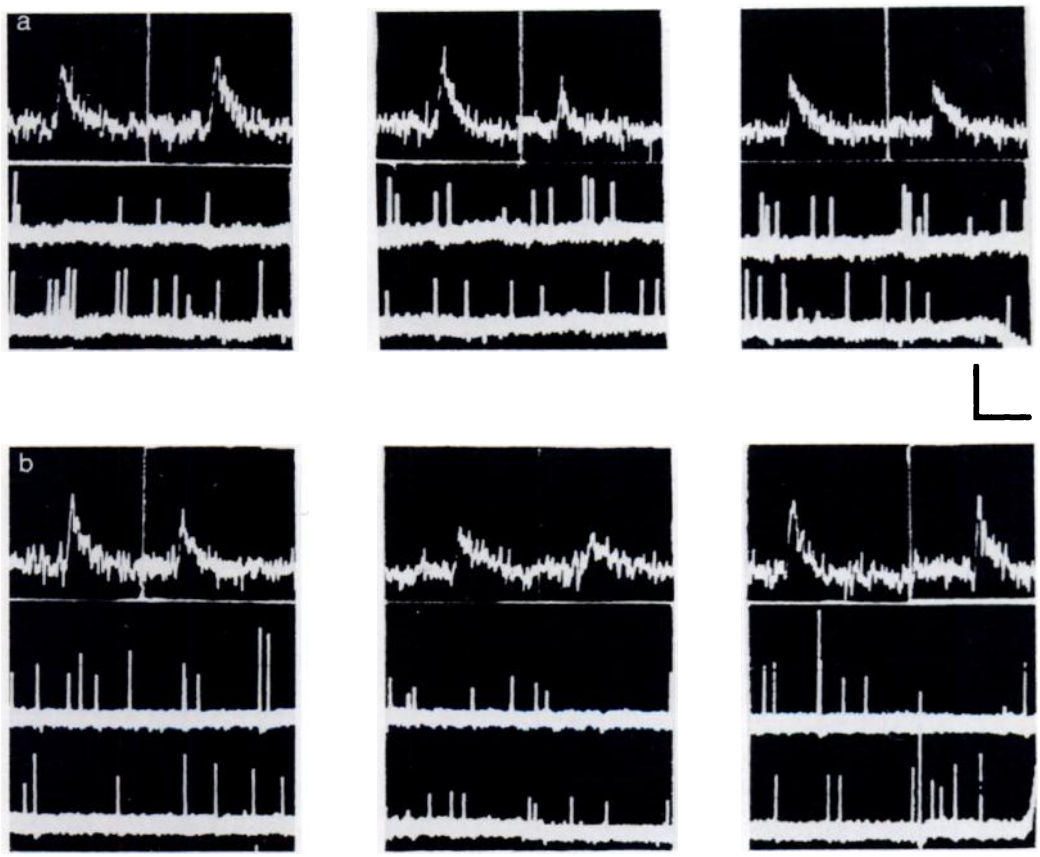

FIG. 3. *The effect of ether (10^{-3} M—a and 10^{-2} M—b.; a, b—two different cells) on the MEPCs*

The arrangement of the sets and traces in this figure is the same as in Fig. 1. At 10^{-3} M the time of decay was shortened by 20% and the amplitude was unchanged (101%). At 10^{-2} M the time of decay was prolonged by 32% and the amplitude decreased by 45%. Calibrations are 2.5 nA and 5 msec for the upper traces (a and b) and 5 nA and 0.5 sec for the lower traces in a and 2.5 nA and 1 sec for the lower traces in b.

(see METHODS, Fig. 1) in six different cells that were exposed each to the control solution, $5 \cdot 10^{-4}$ M methoxyflurane and 10^{-2} M ether. The reversal potential was −4 mV ± 1.2 for control, −3 mV ± 1.6 for methoxyflurane and −5 mV ± 1.6 for ether. It is clear that these drugs do not affect the reversal potential and thus presumably have no effect on the selectivity of the synaptic channels. The reversal potential for calculating the single channel conductance was therefore taken as −5 mV (10–12).

The effects of methoxyflurane and ether on the MEPC are fully corroborated by their effects on the single channel parameters. In normal end-plates, the time-constant of decay of the MEPC equals the mean open time of the single synaptic channel (4). However, the amplitude of the MEPC depends both on single channel conductance and on the number of channels that are activated by a single quantum of transmitter. Conceivably then, the effects of a drug on the amplitude of the MEPC could be mediated by one or a combination of the following: decrease in the affinity of ACh for its receptor and reduced density of receptors at the synapse. To resolve this issue, we performed ACh noise analysis and obtained ACh noise power spectra, from which the mean single channel conductance (γ) and the mean single channel open-time (τ) could be calculated (see METHODS). The effect of methoxyflurane ($5 \cdot 10^{-4}$ M) was studied on 5 cells and that of ether was studied on 3. The control values for all 8 cells were: mean single channel conductance (γ) = 28 ± 2.5 pS and mean open-time (τ) = 3.25 ± 0.1 msec. The holding potential for all cells was −70 mV and the temperature was in the range of 7–10°. Figure 4 shows the effect of methoxyflurane on the ACh noise spectrum. In this experiment τ was shortened from 3.7 msec to 2.1 msec

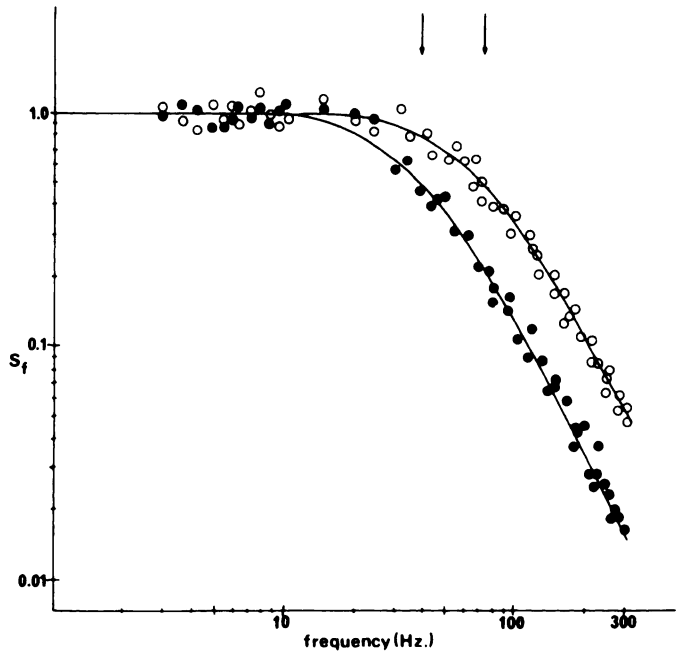

FIG. 4. *The effect of methoxyflurane ($5 \cdot 10^{-4}$ M) on ACh noise*

The spectrum in the presence of methoxyflurane (hollow circles) is shown together with the spectrum at control solution (filled circles). The ordinate is a normalized power density $S(f)$. The values for this cell were: $S(o) = 4.1 \cdot 10^{-22}$ A^2 sec in control and $2.0 \cdot 10^{-22}$ A^2 sec in methoxyflurane, f_c = 43 Hz in control and 75 Hz in methoxyflurane (indicated by arrows), and μ_I = 30 nA in control and 41 nA in methoxyflurane. The calculated parameters were: in control τ = 3.7 msec, γ = 28 pS, and in methoxyflurane τ = 2.1 msec γ 17.8 pS. The temperature was 8° and the holding potential −70 mV.

TABLE 1

The effect of the ethers on the MEPC and single channel parameters

Drug and concentration	Parameter change (as percent of control)			
	MEPC mean amplitude	Single channel conductance (from Ach noise)	MEPC half decay time	Mean channel open time (from Ach noise)
Methoxyflurane				
10^{-4} M	−21% ± 1[a] (n = 5)		−31% ± 2 (n = 5)	
$5 \cdot 10^{-4}$ M	−43% ± 2 (n = 5)	−38% ± 2 (n = 5)	−31% ± 1 (n = 5)	−30% ± 2 (n = 5)
Fluothyl				
10^{-3} M	−32% ± 1 (n = 6)		−32% ± 0.5 (n = 6)	
Ether				
10^{-3} M	+1% ± 0.5 (n = 5)		−21% ± 1 (n = 5)	
10^{-2} M	−45% ± 1 (n = 3)	−42% ± 3 (n = 3)	+35% ± 3 (n = 5)	+35% ± 2 (n = 3)

[a] Mean ± SEM.

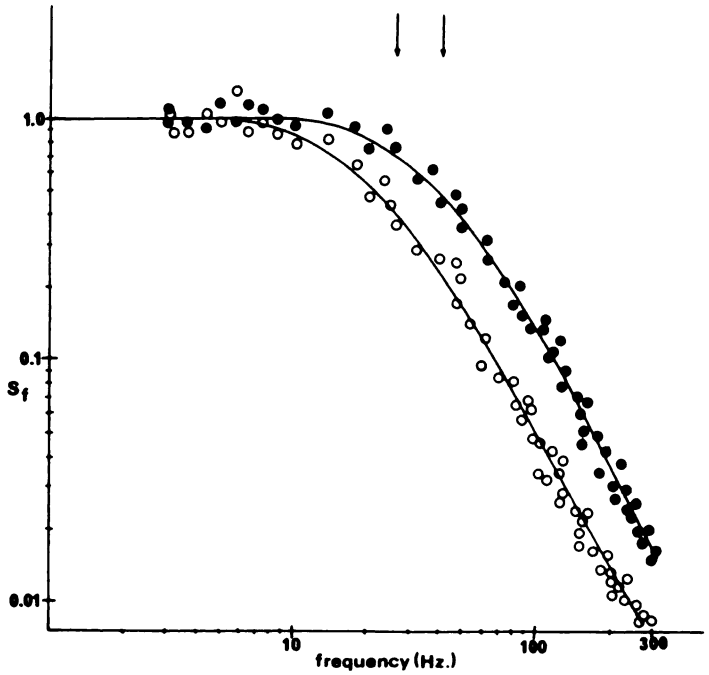

FIG. 5. *The effect of ether (10^{-2} M) on ACh noise*

The spectrum in the presence of ether (hollow circles) and in control (filled circles) are shown. The values in this cell were: $S(o) = 5.3 \cdot 10^{-22}$ A² sec in control and $4.1 \cdot 10^{-22}$ A² sec in ether, $f_c = 44$ Hz in control and 28 Hz in ether (indicated by arrows) and $\mu_I = 35$ nA in control and 38 nA in ether. The calculated parameters were: in control $\tau = 3.65$ msec and $\gamma = 32$ pS and in ether $\tau = 5.6$ msec and $\gamma = 19$ pS. The temperature was 7.5°C and the holding potential −70 mV.

and γ was decreased from 28 to 17.8 pS. The average effect of the drug was to decrease τ by 30% ± 2 and γ by 38% ± 2 (Table 1). Again, there was no evidence for a slow second component as had been demonstrated with local anesthetics (7, 8). Figure 5 shows the effect of ether (10^{-2} M) on the ACh noise spectrum. Here τ was increased from 3.65 to 5.6 msec and γ was reduced from 32 to 19 pS. The average effect was an increase of τ by 35% ± 2 and a decrease of γ by 42% ± 3. If one compares the effects of the ethers on the MEPCs and on the single channel parameters one finds a close correspondence (Table 1). The fact that the MEPCs and ACh noise were each

measured at a different temperature does not invalidate this finding. In this context, we recall that γ is not temperature-sensitive but that τ has a Q_{10} of about 2.8 (4). Still, it is obvious that the relative effect of the ethers on each of these parameters is comparable in both systems. We therefore conclude that the effects of the ethers on the MEPC are fully corroborated by their effects on the single channel properties; for a rationalization of the present results, one does not need to assume changes in ACh affinity, or number of available receptors, or rate of disappearance of ACh (13).

DISCUSSION

Our results afford additional proof that both methoxyflurane and fluothyl share a common mechanism of action in their effect on the end-plate. Thus, the concentration at which either drug reduces MEPP amplitude is also the concentration that causes a decrease in the amplitude and duration of the MEPC (Table 1). If within the framework of the present study the MEPC parameters are considered a measure of the single channel conductance (as Table 1 indicates), then either drug reduces τ as well as γ. This finding is in line with our hypothesis (1) that such structurally non-specific drugs exert analogous effects at their site of action, be they anesthetic as methoxyflurane, or convulsant as fluothyl. If drug-elicited depression of end-plate sensitivity involves in this case a process of entropy gain, then the more membrane-soluble methoxyflurane would appear to be more potent than the less soluble fluothyl, as indeed is the case.

Beyond this conclusion, the present findings also offer an interesting insight into the molecular processes underlying drug action presumed not to be receptor-mediated. All the agents of concern in this work affect both the open-time and single channel conductance of the ACh receptor; but a particular agent seems to exert a differential effect on each of these two functions. Thus, an increase from 10^{-4} to $5 \cdot 10^{-4}$ M in methoxyflurane does not entail further reduction in τ or decay rate beyond 30% but it will further depress MEPC amplitude from 80 to 60%. Thus, the latter function appears to be the more vulnerable one to the effect of the drug. In the absence of a complete dose-response curve, it is impossible to decide whether there is in fact a ceiling to the effect of methoxyflurane on MEPC decay, but the results with ether could be illuminating in this context. Gage and Hamill (9), who were the first to study the effects of ether and enflurane on the MEPC, reported a biphasic effect of the former on the decay rate, first a decrease then an increase in τ that follows a rise in concentration of the drug. Our results with ether confirm the findings of these authors. In addition, they show that ether affects amplitude, i.e., single channel conductance in the same direction as the other two agents studied, but is also much weaker in this respect. Now, if one considers the ceiling in the effect of methoxyflurane as the transition point between a decrease in MEPC decay and a subsequent hypothetical increase, in analogy with the biphasic effect of ether, then the two drugs would seem to follow much the same pattern. The uniqueness of ether in the series is its high water solubility which allows use of bathing solutions as concentrated as 10^{-2} M; the maximum experimental concentrations of the two other agents is almost at the limit of their water solubilities.

On the face of the present evidence, there seems to exist at the level of the post-synaptic membrane at least three functions that may be modified or modulated with structurally non-specific agents: decrease in channel open-time, increase in channel open time and decrease in single channel conductance. That such functions are given to the control of separate membrane entities is suggested by a number of relevant publications (11–14) and by the more recent results of Sobel *et al.* (15). In particular, the effect on single channel conductance deserves special comment. To the best of our knowledge, this is the first work reporting a decrease in single channel conductance under the effect of ethers. The mechanism of this effect is presently unknown. Local anesthetics were shown to reduce single channel conductance by a process of alternating and rapid blocking and unblocking of the channel proper (16). Such a mechanism is expected to lead to the appearance of a second, high-frequency component in

the power spectrum (7, 8); even though such a component could not be detected in the present case (Figs. 4 and 5), it is still possible that such a mechanism is operative also for ethers, but at a rate that is undetectable in the range of our frequency measurements (3–307 Hz). Also, channel-blocking activity seems to ensue from an entropy-gain process, because the effective concentration of ether, the agent with the lowest oil/water partition coefficient, is also the highest. This view is consistent with the findings of Hille (17) implicating lipid-phase solubility in the channel-blocking activity of local anesthetics. Finally, one is tempted to comment on the relevance of the experimental concentrations used in this work to actual effective dosage in anesthesia. The effective anesthetic concentration of ether in blood at stage 3 is about 20 mM which, experimentally, corresponds to a situation in which τ would increase significantly above control values, while γ would decrease by almost 50%. In the range of the anesthetic concentration of methoxyflurane, $3 \cdot 10^{-5}$ M, both γ and τ are expected to decrease, the former much less than the corresponding decrease with ether. It is not unlikely that such differential effects at the most basic level of drug action are also at the root of any differences in the *quality* of drug action *in vivo*.

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