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ANALYSIS OF ATROPINE ACTION AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. Atropine action on the end-plate currents (e.p.c.s) has been analysed at the macroscopic and elementary levels.

2. The shortening effect of atropine on the e.p.c. and m.e.p.c. level can be fully explained by a reduction of the life time of the elementary current: this effect is markedly increased at more hyperpolarized membrane potentials and at higher concentrations of atropine.

3. It is therefore suggested that atropine binds to the open acetylcholine-receptor complex, leading to a state with a null conductance. According to this model, the forward rate constant of atropine binding could be calculated and was of the order of 10^7 M^{-1} s^{-1} at -90 mV and 20–22° C.

4. Although the conductance at the peak of the e.p.c. is reduced by atropine and becomes voltage sensitive, the elementary conductance is affected neither by voltage nor by atropine.

5. The exclusive binding of atropine to the activated ACh-receptor complex, as proposed above, does not appear to explain this phenomenon. Another binding occurring before the channel is open with a dissociation constant of 60 μM could account for this effect.

INTRODUCTION

Under normal conditions, the action of acetylcholine (ACh) on the nicotinic receptor of the frog skeletal muscle end-plate is voltage dependent, with longer synaptic responses found at more hyperpolarized membrane potentials. This voltage dependence was first shown on the end-plate potential (e.p.p.) and end-plate currents (e.p.c.) (Takeuchi &

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Takeuchi, 1959; Kordaš, 1969; Magleby & Stevens, 1972*a*, *b*), and more recently Anderson & Stevens (1973) have shown that it is also a characteristic of the elementary ACh current. This voltage sensitivity could be related to a property of the ionic channels which are gated by an electric field sensor, or could reflect a voltage dependence of the ACh binding site (Magleby & Stevens, 1972*a*, *b*). An analysis of the voltage dependence of the effects of various agonists and antagonists may provide information on the site of action of the electric field.

Antagonists of the competitive type, such as curare and bungarotoxin, do not affect the elementary current (Katz & Miledi, 1972, 1973*c*) and therefore do not provide a clue to the question we ask. On the other hand, Katz & Miledi (1973*b*) have shown that a non-competitive antagonist like atropine shortens the elementary effect of ACh, and for this reason an understanding of the site of action of atropine would be particularly relevant.

Atropine has a dual action at the end-plate of frog skeletal muscle. It shortens the time course and it reduces the amplitude of the e.p.p. (Beránek & Vyskočil, 1968) and of the e.p.c. (Kordaš, 1968; Magazanik & Vyskočil, 1969). In addition, simultaneous reports of the action of the atropine on the e.p.c.s (Adler & Albuquerque, 1976) and on the miniature end-plate currents (m.e.p.c.s) (Feltz & Large, 1976) have pointed out that atropine reduces the voltage sensitivity of the decay of these currents in a dose dependent fashion.

At the molecular level, Katz & Miledi (1973*b*) have shown that the shortening effect of atropine was due to a reduction of the life time of the elementary current. These authors pointed out that further investigations were necessary to decide whether atropine had other effects at the end-plate, namely if it affected the elementary conductance and/or the affinity constant for ACh.

In the present paper, we have used the technique of noise analysis (Katz & Miledi, 1972; Anderson & Stevens, 1973) to examine the two parameters of the elementary current (mean amplitude and duration) as a function of atropine concentration, voltage and temperature. From the data we have obtained, quantitative comparisons can be made between the effect of atropine on the end-plate currents at the elementary and macroscopic levels.

We conclude that atropine reduces both the mean life time of the channels and the probability that they open, while the elementary conductance remains unaltered. Furthermore, we discuss several models and our tentative conclusion is that atropine binds both before and after the opening of the channel.

METHODS

Experiments were performed on the cutaneus pectoris and the sartorius muscles of the frog, *Rana esculenta*. The preparation was constantly superfused, at a rate of 3 ml./min in a 2 ml. volume, by a Ringer of the following composition (mM): NaCl, 115; $CaCl_2$, 1·8; KCl, 2·5; Tris maleate, 2; acetylglycine, 2. The pH was adjusted to pH 7·2 by addition of NaOH. The bath was cooled and temperature was monitored continuously by a thermistor. Atropine sulphate (Serlabo) was diluted in the bathing fluid at the stated concentrations and its effects studied after at least 10 min of superfusion. In the study of m.e.p.c.s, the NaCl concentration was brought up to 161 mM to increase the osmotic pressure and hence the frequency of the m.e.p.p.s. In the experiments in which the nerve was stimulated, the quantum content was decreased by replacing NaCl isosmotically by 9–10 mM-$MgCl_2$. For noise analysis, ACh was applied by iontophoresis in the vicinity of the end-plate from a 2 M-ACh filled electrode so as to produce not more than a 200 nA response current.

Voltage clamp technique

Both voltage and current passing electrodes were filled with 3 M-KCl (resistance of 1–5 MΩ in normal Ringer solution) and end-plates were located by recording m.e.p.p.s. with a fast rise time ($< 1·5$ ms) and the current electrode was then inserted into the cell within 100 μm of the recording electrode. At room temperature, m.e.p.c.s had a rise time of about 0·5 ms.

In voltage clamp conditions, the gain of the output amplifier of the feed-back system was adjusted so that a discrepancy of less than 5% was observed between the actual voltage and an imposed DC-voltage step. The ACh-induced current was estimated from the voltage drop across a 1 MΩ resistor in series with the current passing electrode. The voltage changes were usually imposed in 10 mV steps. When fibres were depolarized, contraction was avoided by applying a very progressive depolarization in 1 mV steps around the spike threshold. Local contractions damaging the membrane around the electrode were certainly not avoided by this procedure since on return to control potential, larger clamp currents were required.

Measurements of the decay time of the m.e.p.c.s and e.p.c.s

The time course of the synaptic currents was measured on enlarged photographic recordings of oscilloscopic displays, taken with moving film for the m.e.p.c.s and on single frames for the e.p.c.s.

The decays of the current $I(t)$ fitted the equation $I(t) = I(t = 0)\, e^{-t/\tau}$, where τ is decay time constant. So $I(t)$ was plotted semilogarithmically on a Hewlett–Packard desk computer and an equally weighted straight line fitted to estimate τ. For each measurement, the decay constants of at least six synaptic currents were calculated and averaged for each parameter. For m.e.p.c.s, unless stated, the mean $\pm$ 2 S.E. of the decay time constant was calculated from at least six cells for each experimental condition. The mean decay times of m.e.p.c.s were over-estimated (15–25%) since they were measured on a rolling film, with an oblique base line. In the results, unless specified, only corrected values are given.

Noise analysis

The end-plate current fluctuations, filtered through a low pass filter (set to 200, 500 or 800 Hz; active Butterworth filter, 48 db/decade) to avoid 'aliasing' errors during analysis, were recorded simultaneously on a low gain DC channel and on a

high gain AC channel. The current fluctuations were fed into a computer (Digital Equipment, PDP8) to be digitalized and the noise power spectra were established according to a FFT programme. The power spectra were analysed over a range of 2–250 Hz for the action of ACh at low temperature, 2–500 Hz for the action of ACh at temperatures above 10° C, and 2–1000 Hz for the action of ACh in presence of atropine at any temperature. The sampling was performed using respectively 256, 512 or 1024 points, each scan lasting 510 ms. Four to ten power spectra were averaged to establish a power spectrum. The characteristic spectrum of ACh were obtained after a subtraction of the background noise, which is justified if the background sources are uncorrelated with the ACh fluctuations (Anderson & Stevens, 1973). The spectrum was finally established in log-log co-ordinates.

The power spectrum was fitted with a single Lorentzian curve, i.e. $S(f)=S(0)/1+(f/f_c)^2$, were f_c is the cut-off frequency, at which $S(f)$ has decreased to half of its zero frequency asymptote. If it is assumed that

(1) the total current evoked by ACh results from continuous fluctuations of the end-plate channels between open and closed states;

(2) the fluctuations occur at random intervals; and

(3) the ACh concentration is low relative to the receptor density (Anderson & Stevens, 1973) so that the underlying event follows a Poisson distribution, it is then possible to estimate the two parameters of the elementary event.

Its mean duration, τ noise, will be $\tau = 1/(2\pi f_c)$ (Katz & Miledi, 1972; Anderson & Stevens, 1973). The conductance γ was estimated from $\gamma = S(0)/2\,\mu_i\,(V-V_0)\,\tau$ noise, where $S(0)$ is the plateau value of the spectrum in the low range frequency, μ_i the total ACh current, and $V-V_0$ the driving force. Artifacts in the extreme ranges of frequency were distinguishable on the spectrum.

RESULTS

Effect of atropine on the duration of the synaptic events

Synaptic currents in normal conditions

We compared the m.e.p.c. decay time ($\tau_{m.e.p.c.}$) and the mean life time of the channel activated by ACh (τ_{noise}), and found that, at 8° C and −90 mV, the mean values of $\tau_{m.e.p.c.}$ and τ_{noise} are similar (6 ms). This first result is in accordance with the observations of Anderson & Stevens (1973) who found a striking similarity between the two values on the same cell.

In contrast, at 21° C and −90 mV, we found values of $1{\cdot}02 \pm 0{\cdot}22$ ms for τ_{noise}, $1{\cdot}76 \pm 0{\cdot}10$ ms for $\tau_{m.e.p.c.}$ (corrected value, see Methods), and $1{\cdot}41 \pm 0{\cdot}22$ ms for $\tau_{e.p.c.}$ Thus the duration of the elementary event appears to be shorter than $\tau_{e.p.c.}$ or $\tau_{m.e.p.c.}$

Identical values for the different τs are only expected when the time during which ACh is present in the cleft is much shorter than the mean life time of the ionic channels. One interpretation of the observed difference between τ_{noise} and $\tau_{m.e.p.c.}$ at high temperatures is that increasing the temperature accelerates the closure of the ionic channel more than it accelerates the disappearance of ACh from the cleft. If this is so, τ_{noise} and $\tau_{e.p.c.s.}$ values are indeed expected to be more similar at lower temperatures.

In addition, removal of the ACh from the cleft may be impeded by the repetitive binding described by Katz & Miledi (1973*a*), so that the decay time of the m.e.p.c. is a function not only of the channel life time but of the number of successive bindings in which a single ACh molecule is involved. Katz & Miledi (1973*a*) observed, after blockade of acetylcholinesterase (AChE), a significant shortening of the decay phase of the e.p.c. by curare. They have attributed this effect to the fact that, when AChE activity was

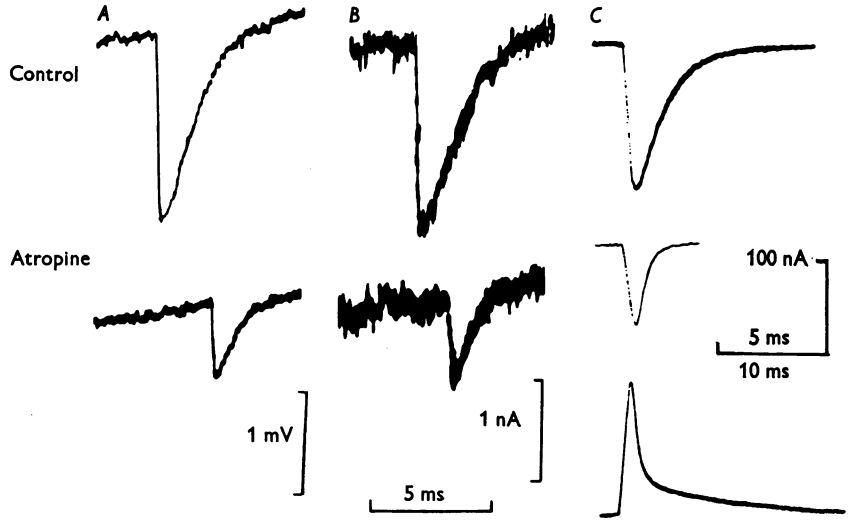


Fig. 1. Effect of atropine on responses to ACh release: spontaneous release (*A*, *B*, effect of $2{\cdot}5 \times 10^{-5}$ M atropine) or nerve-evoked release (*C*, effect of 5×10^{-5} M atropine). The control responses (upper row) can be compared to the responses in the presence of atropine (lower row). The shortening effect of atropine can be observed on extracellular m.e.p.c.s. (*A*) and on intracellular current recorded in voltage clamped fibre (*B*, holding potential at -90 mV; *C*, holding potential at -80 mV). In the lower corner at the right is given a picture in presence of atropine of the end-plate potential at the resting potential of -60 mV. It shows a clearly biphasic decay which is much less apparent on the current (same end-plate for *C*, but different time scale for current and potential).

blocked, a single ACh molecule could combine successively to different receptors, therefore lengthening the time of ACh presence in the cleft. In the presence of curare, the probability that an ACh molecule hits a free receptor is reduced, as is the number of ACh bindings before its removal from the cleft; as a result, the e.p.c. decay is shortened. We have observed that even without anti-AChE, $\tau_{e.p.c.}$ is reduced by curare (10% for curare 2×10^{-6} g/ml.; see also Molgo, 1975). In general, the values of $\tau_{e.p.c.}$ reported in the literature are shorter in the presence of curare than

when the quantal content was reduced by high Mg^{2+}. These data suggest that, at 21° C, and in the absence of any AChE inhibitors, the removal of ACh from the cleft is slightly slowed down by ACh binding. Katz & Miledi (1973*a*) who observed that $\tau_{m.e.p.c.}$ (from extracellular recordings) was 1·5–2 times longer than τ_{noise}, assumed that in normal conditions some ACh molecules survived in the cleft after one or two receptor occupations.

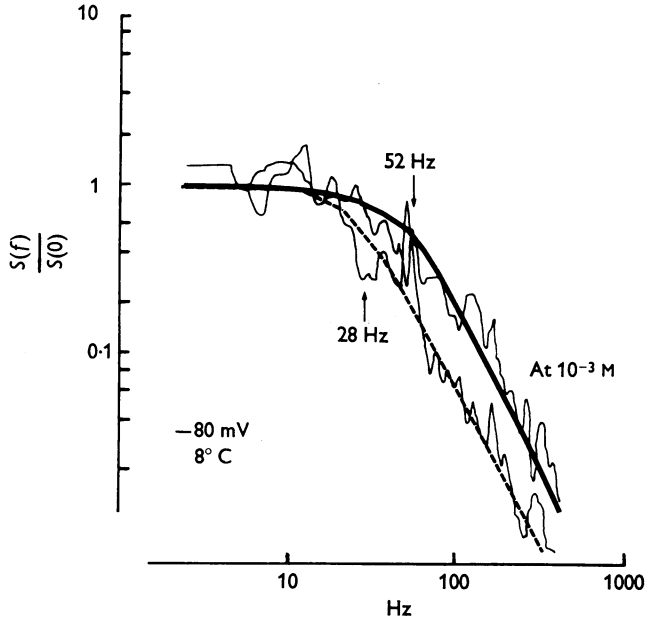


Fig. 2. Effect of atropine on the spectral density of current fluctuations produced by a steady iontophoretic application of ACh to a voltage clamped end-plate, expressed in relative units. A Lorentzian curve has been fitted by eye to each spectrum (interrupted line for the control, and continuous line in presence of atropine). Arrows indicate the cut-off frequencies. The spectra are based on 5120 digital samples taken at 2 kHz through a 800 Hz active low pass filter. The resulting spectra (512 points) have been smoothed (weighted mean of the ten nearest points), and then drawn as a continuous line on a *X–Y* plotter.

Effect of atropine concentration on the duration of the synaptic current

Atropine shortens the duration of the synaptic currents, as it has been described for the e.p.c. (Beránek & Vyskočil, 1967; Kordaš, 1968; Adler & Albuquerque, 1976) and for the elementary event (Katz & Miledi, 1973*b*). Figs. 1 and 2 show this effect of atropine on m.e.p.c. and e.p.c., and on the spectrum of ACh noise. It can be seen (Fig. 2) that in 10^{-5} M atropine, at 8° C and −80 mV, the life span of the elementary current

induced by ACh is shortened from 5·7 ms (cut-off frequency = 28 Hz) to 2·85 ms (55 Hz). A similar shortening was observed for the e.p.c. For this e.p.c., close analysis of the decay shows that it is biphasic, the initial steep peak being prolonged by a tail of relatively small amplitude (Fig. 1). This tail, though never mentioned, can be observed in records of other authors (Beránek & Vyskočil, 1968, Fig. 2; Magazanik & Vyskočil, 1973, Fig. 1). At −90 mV and 21° C, it has a time constant of decay of 6 ± 3 ms (S.D.) and does not exceed 5% of the total current when extrapolated to zero (peak) time. But its relative contribution increases significantly with

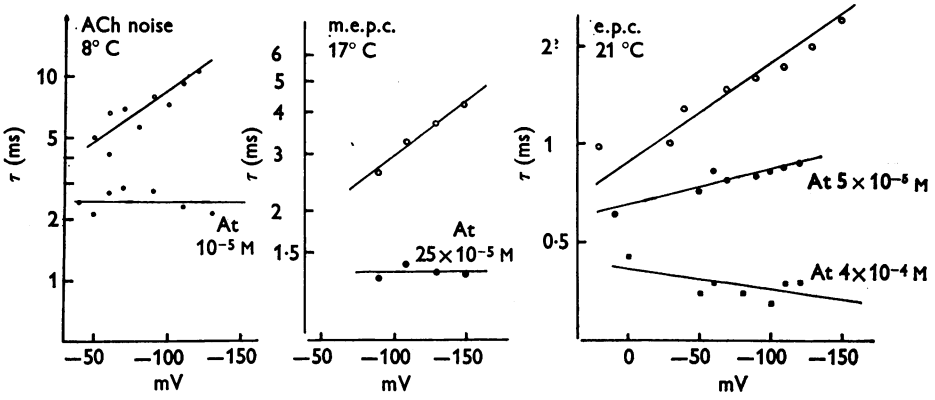


Fig. 3. Effect of voltage on the duration of end-plate currents, from left to right τ_{noise}, $\tau_{m.e.p.c.}$, and $\tau_{e.p.c.}$, before (open circles) and after (filled symbols) addition of atropine. Measurements were done on three different end-plates at 8, 17 and 21° C respectively. Other data obtained from the end-plate used for $\tau_{e.p.c.}$ are shown on Figs. 5 and 8. Lines represent least-squares fits.

depolarization. Because of its small amplitude and slow time course, the process is measured in an inaccurate way in the e.p.c., and escapes analysis in m.e.p.c.s, or in a noise spectrum. It will therefore not be discussed in the analysis of our experimental results. We will just note here that, although the appearance of a slow tail could result from a specific effect of atropine (see Discussion), one cannot exclude that it is due to a complex release function of ACh. In fact, we have frequently observed in *normal* e.p.c.s a prolonged tail which could be explained by a transmitter release persisting for a number of milliseconds (Katz & Miledi, 1967; Barrett & Stevens, 1972). In such a case, the shortening effect of atropine on the elementary event could shorten the initial phase of the response without altering the slow phase (related to an asynchronous release of ACh). The differences in the slope of the two phases would then be emphasized.

Increasing the atropine concentration causes an increased shortening of the synaptic currents (Fig. 3). Similar dose–response curves were obtained for τ_{noise}, $\tau_{m.e.p.c.}$ and $\tau_{e.p.c.}$, with a shortening to half the control value in presence of 2×10^{-5} M atropine (Fig. 4). One may compare this value with the one describing the effect of atropine on the e.p.c. amplitude. From Beranek & Vyskočil (1967) and Adler & Albuquerque (1976), the apparent concentration for the e.p.c. reduction is 6×10^{-5} M (Fig. 4). For atropine concentrations above 10^{-4} M, $\tau_{e.p.c.}$ decreases by a further 20% of the initial value, whereas the amplitude of the e.p.c. shows a greater reduction (by 40%).

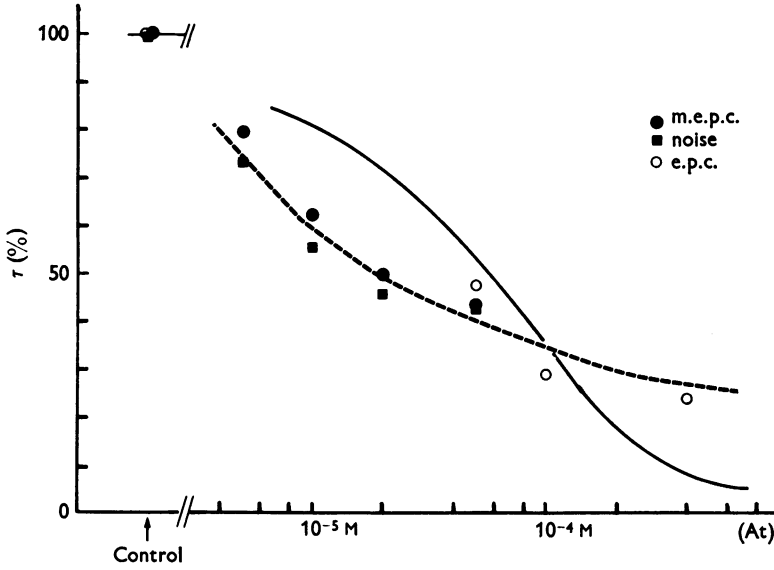


Fig. 4. Dose–response curve of the duration of the end-plates currents ($\tau_{e.p.c.}$, $\tau_{m.e.p.c.}$, τ_{noise}) under various concentrations of atropine at 21° C and −90 mV. All durations are expressed as percentages of the controls values which were respectively 1·43, 1·76 and 1·02 ms. Interrupted line drawn by eye. For comparison, the effect of atropine on the e.p.c. amplitude is given by the continuous line. This line has been drawn from the pooled data of Berănek & Vyskočil (1967) and Adler & Albuquerque (1976).

Atropine action and loss of voltage sensitivity of the ACh currents

Increasing concentrations of atropine cause a gradual loss of the voltage sensitivity of the e.p.c.s (Adler & Albuquerque, 1976) and of the m.e.p.c.s (Feltz & Large, 1976), the voltage sensitivity being totally abolished for high concentrations. We have shown that atropine affects τ_{noise} in a similar way. Fig. 3, where the variations of τ_{noise}, $\tau_{m.e.p.c.}$ and $\tau_{e.p.c.}$ are plotted against the membrane potential, illustrates this striking effect of

atropine. At 21° C, the voltage sensitivity of an e.p.c. decay time (Fig. 3, on the right) was much reduced in 10^{-5} M atropine, and was totally abolished for atropine concentrations between 5×10^{-5} and 10^{-4} M. By further increasing the atropine concentration, $\tau_{e.p.c.}$ was further reduced, and in some cases a slight reversal voltage sensitivity appeared (Fig. 3; 4×10^{-4} M atropine).

The concentrations of atropine necessary to abolish the voltage sensitivity of τ_s were 10^{-5} M for noise, $2{\cdot}5 \times 10^{-5}$ M for m.e.p.c. and 5×10^{-5} M for e.p.c. We do not know if the differences in these values established in different experiments are meaningful.

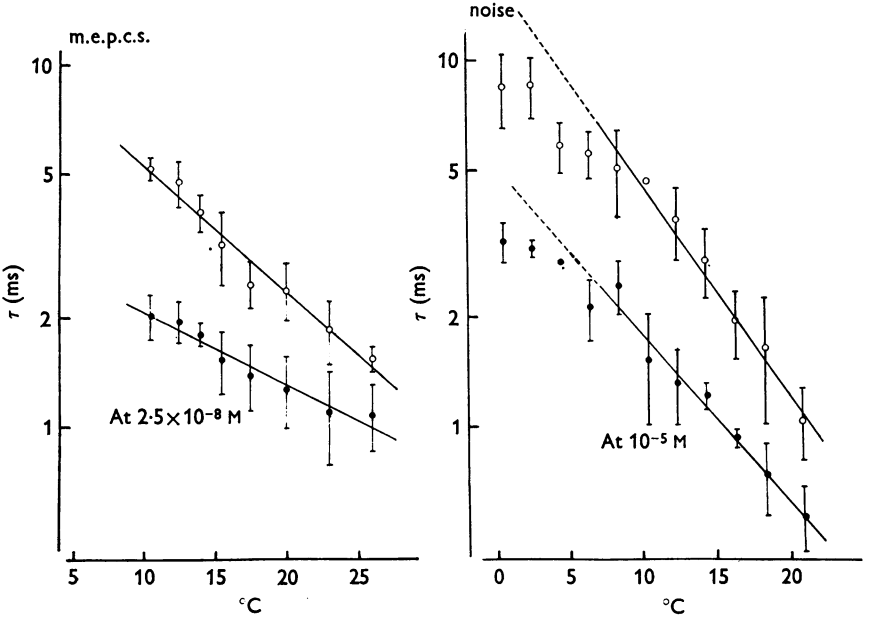


Fig. 5. Temperature dependence of the mean life time of the ionic channel (right) and of the decay time of m.e.p.c.s. (left). Open circles: control; filled circles: after addition of atropine. The $\tau_{m.e.p.c.s}$ values have not been corrected for the artifactual lengthening due to the recording method on rolling film (see Methods). Each point is the mean of at least six measurements for $\tau_{m.e.p.c.}$ (data pooled from twenty cells) and at least three measurements for τ_{noise} (data from ten cells in presence of atropine, among which four were used in the normal conditions). Vertical bars represent ± S.D. The continuous lines are regression lines fitted by the least-squares method (data above 8° C were only used for τ_{noise}).

A consistent observation was also that the rise time of the e.p.c. behaves in a similar way to that of the decay time: under normal conditions it is lengthened by hyperpolarization. In the presence of atropine, it is shortened and loses its voltage sensitivity.

Atropine effect on temperature dependence of synaptic currents

It is well known that the duration of the synaptic current is lengthened when the temperature is decreased. By using the Arrhenius equation, an activation energy can be calculated at the resting potential. The values of the activation energy for the $\tau_{m.e.p.c.s}$ reported by various authors range from 14 kcal/mole (Molgo, 1975) to 18 kcal/mole (Gage & McBurney, 1975). Our measurements gave a value of 13·5 kcal/mole (Fig. 5, left).

For τ_{noise}, the reported values range from 11 kcal/mole (Ben Haim, Dreyer & Peper, 1975) to 17 kcal/mole (Anderson & Stevens, 1973). In our experiments, by changing the temperature from 1 to 22° C, we observed a break in the Arrhenius plot for τ_{noise} around 8° C. Below 8° C, the temperature dependence for τ_{noise} is less marked than above 8° C. If an activation energy of 16 kcal/mole can account for the temperature dependence over the entire temperature range, for the measurements above 8° C a much higher activation energy (22·6 kcal/mole) is calculated (Fig. 5, right).

The break in the temperature curve for τ_{noise} is puzzling. Its direction is unexpected and excludes the possibility that it is due to a phase transition in the lipids, 'freezing' the membrane (Lass & Fischbach, 1976). If at low temperatures, the closing of the channels was governed by another rate limiting step, it would have been revealed by a break in the curve in the other direction.

In presence of atropine 10^{-5} M, if one compares results obtained for m.e.p.c.s and noise over a similar range of temperature, i.e. above 8° C, the energy of activation for τ_{noise} and for $\tau_{m.e.p.c.}$ is 5–6 kcal/mole less than in the controls.

The effect of atropine on the conductance of synaptic events

Ionic selectivity of the end-plate channels

We have observed that, for the e.p.c. and the responses to ACh application, the value of the reversal potential remains the same (0 to -10 mV) whether atropine is present or not (Fig. 6). Our interpolated measurements are in accordance with those of Adler & Albuquerque (1976). The observations of Magazanik & Vyskočil (1969), who found that atropine changed the extrapolated value of the reversal potential of the end-plate responses evoked by nerve stimulation, but not for the ACh iontophoresis, are probably accounted for by the difference in the duration of the ACh presence in these two situations. As pointed out by Dionne & Stevens (1975) (see also Adams, 1976; Mallart *et al.* 1976; Trautmann & Zilber-Gachelin, 1976), the voltage dependence of the peak conductance is imposed by the time of presence of ACh in the cleft, rather than by the

life time of the elementary event. One can therefore assume that atropine does not affect the selectivity of the ionic channel opened by ACh.

The effect of atropine on the elementary conductance

The inhibitory effect of atropine is not associated with a change in the elementary conductance γ. It can be seen in Fig. 7 that the elementary

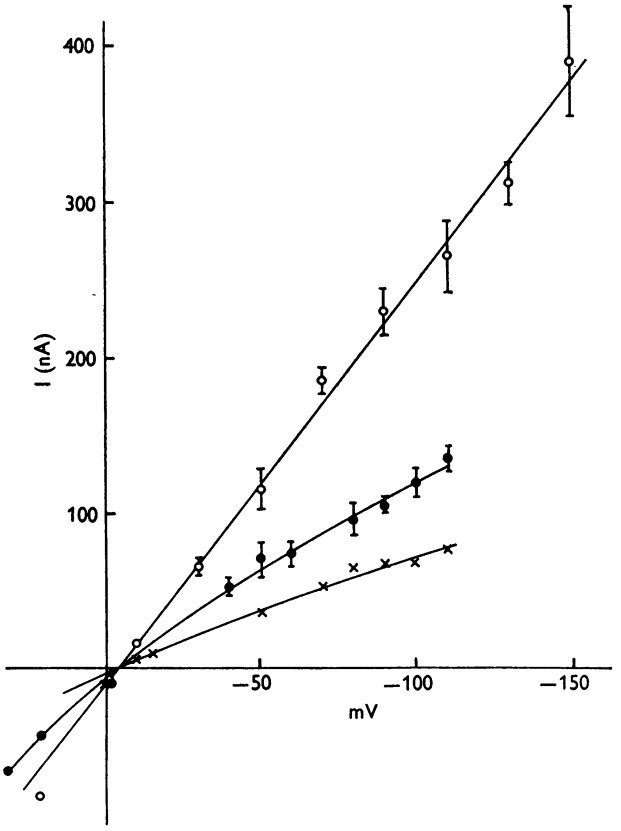


Fig. 6. Effect of voltage on the amplitude of the e.p.c. before (○) and after addition of atropine 5×10^{-5} M (●) and 4×10^{-4} M (×). Same cell as in Fig. 2, right. Each point represents the mean of at least four measurements. Vertical bars give ± S.D. Standard deviations of less than 3 nA were omitted. The lines are drawn according to a linear regression for the control and exponential in presence of atropine. Note that the reversal potential is unchanged by atropine. The estimated underlying conductance is shown in Fig. 8. Temperature 21° C.

conductance is not only independent of voltage but also of atropine concentration. In this Figure, the results for different cells before and after atropine perfusion are pooled. For each cell, the conductance

at different membrane potentials and different atropine concentrations is expressed as a percentage of the mean γ value obtained in the absence of atropine for this cell. It can be seen that there is a large scatter in the individual measurements, but that there is no tendency for either membrane potential or the presence of atropine to affect γ.

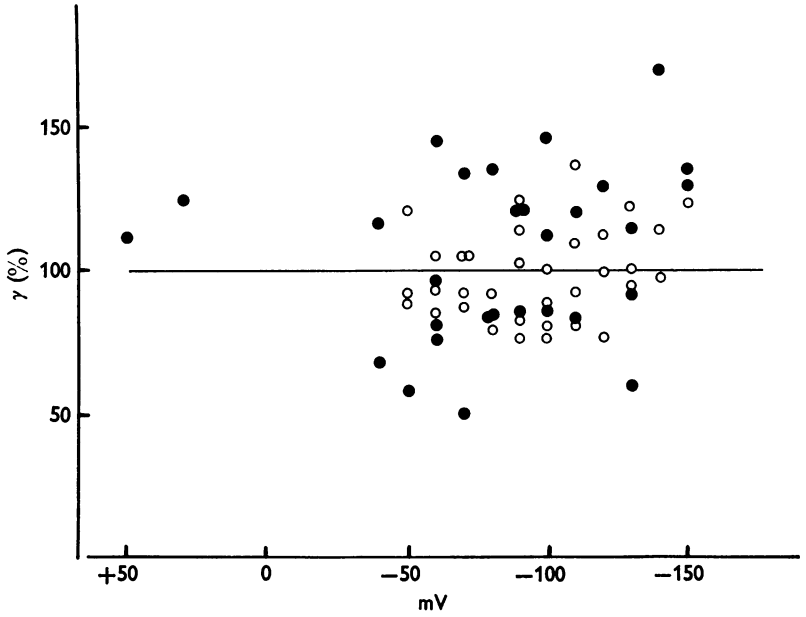


Fig. 7. Effect of voltage on γ, the elementary conductance, before (○) and after addition of 10^{-5} M atropine (●). Data are pooled from five end-plates. Temperature range: 6–12° C. For each end-plate, the conductances are expressed as percentages of a reference, chosen as the mean value of γ before the addition of atropine. There is a rather large scatter of the γ values, but no clear effect of voltage or atropine appears.

The effect of temperature on the value of γ is represented in Fig. 8 which shows a temperature dependence of γ which is unaffected by the presence of atropine. The Q_{10} (between 10 and 20° C) is 1·8. This temperature dependence of γ contrasts with the data of other authors who, in the same preparation, found no temperature dependence for γ (Anderson & Stevens, 1973; Dreyer, Walther & Peper, 1976) or only a slight increase of γ with temperature (Neher & Sakmann, 1976). The dependence that we observed might be over-estimated for two reasons. First, undetected m.e.p.c.s (more frequent at higher temperature unless special care is taken to avoid them: Dreyer *et al.* 1976) may have shifted the spectrum on the ordinate, without greatly changing the cut-off frequency. Secondly, if the spectrum arises from two populations of receptors, as suggested by

Dreyer *et al.* (1976), fitting a single Lorentzian curve to the power spectrum might have introduced variable over-estimates of the conductance associated with the faster component. Therefore we consider that our estimates of γ at low temperatures are likely to be the most reliable. From data obtained between 5 and 10° C, the value of γ was found to be

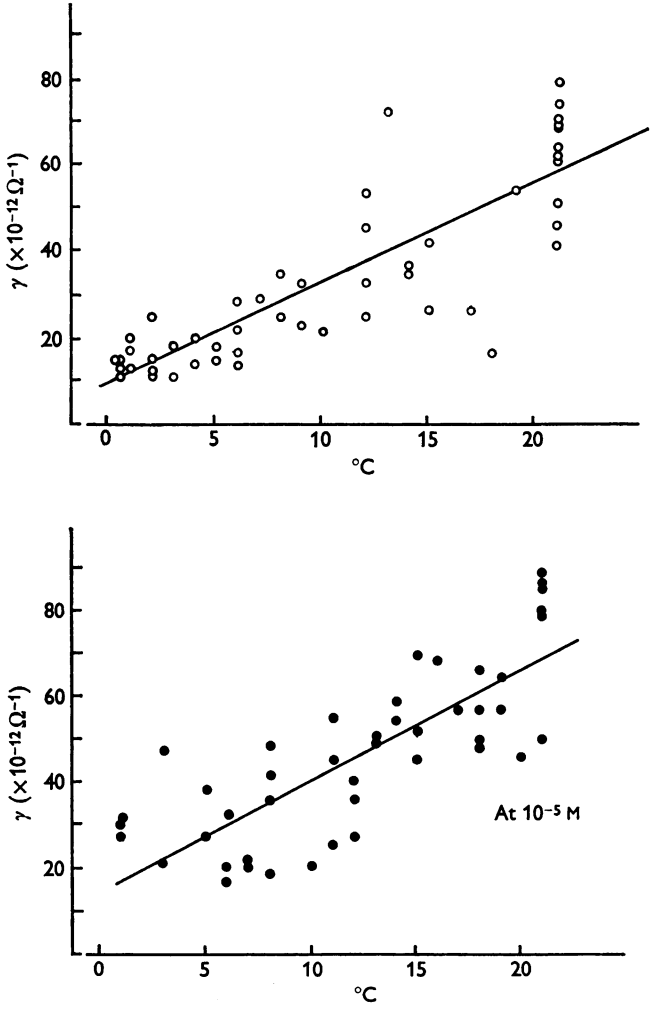


Fig. 8. Estimates of the elementary conductance γ at various temperatures. On top, open circles: control conditions, fifty estimates from eighteen different cells. Bottom, filled circles: in presence of 10^{-5} M atropine, forty-five estimates on seventeen different cells. Lines are least-squares fits using γ ($\times 10^{-12}\,\Omega^{-1}$) $= 10 + 2{\cdot}35\theta$ for control and $\gamma(\times 10^{-12}\Omega^{-1}) = 14{\cdot}2 + 2{\cdot}6\theta$ for atropine, θ being the temperature in ° C.

24 ± 7 pS ($n = 12$; $\pm$S.D.) in normal conditions and 28 ± 10 pS ($n = 12$; $\pm$S.D.) in the presence of 10^{-5} M atropine.

The effect of atropine on the conductance at the peak of the e.p.c.

It has been shown above that in the presence as well as in the absence of atropine, the elementary current is linearly related to the membrane potential; in other words, the elementary conductance is not dependent on the membrane potential.

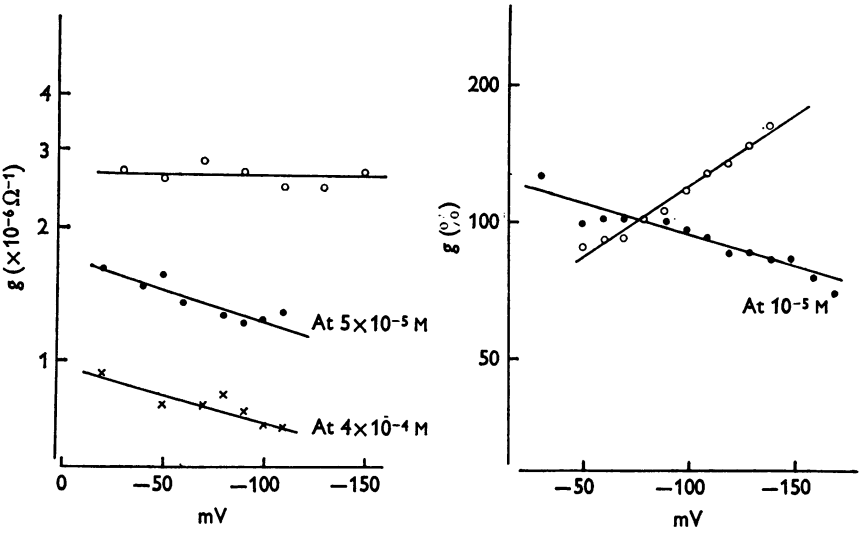


Fig. 9. Effects of voltage on end-plate conductances at the peak of the e.p.c. (left) and during a steady application of ACh (right) before (○) and after addition of atropine (●, ×). It is shown that under atropine, the conductance decreases with hyperpolarization. The data on the left graph come from the same cell as that for Figs. 2 (right) and 5. For the right graph, the currents were measured as described in the text and the reversal potential used to calculate the conductances was arbitrarily set at -10 mV. Measurements have been done in presence of 9 mM-$MgCl_2$ to reduce the quantum content, except when the atropine concentration was 4×10^{-4} M, in which case no $MgCl_2$ was added.

Under normal conditions, we found that the e.p.c. amplitude was also linearly related to the membrane potential, which means that the underlying conductance ($g_{\text{e.p.c.}}$) is also independent of the membrane potential.

On the other hand, in the presence of atropine, the curve relating the e.p.c. amplitude to membrane potential shows a marked flattening when membrane potential is increased, indicating a reduction of g e.p.c. by hyperpolarization. One can see in Fig. 9 that the voltage dependence of $g_{\text{e.p.c.}}$ is identical in 5×10^{-5} and 4×10^{-4} M atropine, though the absolute values were markedly reduced when going from 5×10^{-5} to 4×10^{-4} M.

The effect of atropine on the end-plate current in steady-state conditions

When one estimates the effect of voltage on the current evoked by a maintained iontophoretic application of ACh, one must take into consideration a possible bias due to desensitization (Katz & Thesleff, 1957).

One way to get rid of this bias is to express ACh currents as percentages of the ACh current measured at a reference potential (the holding potential). A step potential is imposed before and during a prolonged ACh application, bringing the membrane potential to the 'test potential'. The difference between the current readings at this 'test potential' is an estimate of the ACh current at this potential. If during each iontophoretic application, a different step is imposed (from always the same reference potential), one may express the ACh currents at each 'test potential' as percentages of the ACh current measured a few milliseconds before at the reference membrane potential.

It is known (Dionne & Stevens, 1975) that for sufficiently slow iontophoretic applications of ACh, the current is not linearly related to the membrane potential: the $I-V$ curve shows a marked curvature, which means that the underlying conductance increases with membrane potential. In the presence of atropine, the $I-V$ curve shows the opposite curvature: the conductance decreases when the membrane potential is increased (Fig. 9, right).

DISCUSSION

As was shown first by Katz & Miledi (1973*b*) the shortening of τ_{noise} produced by atropine explains the shortening of the e.p.p. (Beránek & Vyskočil, 1968; Kordaš, 1968).

The shortening of $\tau_{m.e.p.c.}$ and $\tau_{e.p.c.}$ by atropine is much more marked at hyperpolarized membrane potentials and is increased by increasing atropine concentration (Feltz & Large, 1976; Adler & Albuquerque, 1976). Our results show that these effects are also found when studying the mean channel life time: the shortening of τ_{noise} by atropine is also increased at high membrane potential and high atropine concentrations.

On the other hand, although atropine reduces the conductance at the peak of the e.p.c. and renders it voltage sensitive, it has no effect on the elementary event.

In the following discussion, we explain why we feel that two atropine bindings are necessary to account for the full effect of atropine.

Mean open time of the channel

At first glance, the shortening of e.p.c. and m.e.p.c. by atropine resembles that provoked by a number of other non-competitive antagonists,

and for which two main classes of interpretation have been proposed (see Gage, 1976). The first class is the sequential model which was first considered by Steinbach (1968*a*, *b*) in his study of the effects of lidocaine derivatives. In this model, the non-competitive antagonist has a preferential binding to the activated ACh receptor, and the blocking effect results from a shortening of the life time of the open channel. This model has been used, with various changes, for the numerous drugs which give a procaine-like effect, that is, an inhibitory action mainly expressed by a shortening of the peak of the e.p.c., followed or not by a slow component. These include procaine and lidocaine derivatives (Steinbach, 1968*a*, *b*; Kordaš, 1970; Adams, 1975; Katz & Miledi, 1975*a*, *b*; Ruff, 1976), DFP (Kuba *et al.* 1974), histrionicotoxin (Albuquerque, Kuba & Daly, 1974), barbiturates (Seyama & Narahashi, 1975; Adams, 1976), piperazine (Kuba, Chikazawa & Koketsu, 1976); see also Marty, Neild & Ascher (1976).

Another interpretation of the effect of the above-mentioned compounds, however, has recently been suggested by Beam (1976*a*, *b*). In this alternative, 'parallel' model, the blocking agent would act before the channel is open, leading for example to a population of 'fast channels' in addition to the normal ones. This leads to the prediction (compatible with our results) that the e.p.c. will show a fast component and a normal one. When discussing the effects of the two lidocaine derivatives QX 222 and QX 314, Beam further draws attention to a possible QX 314-like effect of atropine at the end-plate. We think that our observations, in particular the increased shortening of the synaptic responses observed at increasing atropine concentrations, support more easily a sequential model than a parallel one. A prediction of the 'parallel' model in its simplest form is that increasing the atropine concentration will increase the relative importance of the fast component but should not change its rate of decay. To account for the increase of the e.p.c. rate of decay with increasing concentrations of atropine, one needs an additional hypothesis, i.e. that the closing rate itself is dependent upon atropine concentration. On the contrary, if atropine acts on the activated complex (sequential model), leading to a state with a much weaker conductance (or even null) no additional hypothesis is needed to explain the effect of atropine concentrations on the rate of decay of the e.p.c.

The simplest version of the sequential model involves three successive reactions: (1) the binding of ACh (A) with the receptor, leading to a complex AR which (2) in turn allows activation of the channel (AR*); (3) the binding of atropine (At) occurs on the receptor in the activated state

$$A + R \underset{(1)}{\rightleftharpoons} AR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR^* \underset{b}{\overset{+At \quad f}{\rightleftharpoons}} AR^*\,At \quad (2) \quad (3)$$

With regard to the two first steps, a usual approximation is to assume that reaction (1) is at equilibrium, and that the number of activated receptors is small as compared to the total number of receptors. If in addition, $\beta \times (AR)$ and b are small compared to α, an approximation of the life time of the channel in the presence of atropine is given (see Adams, 1976) by $\tau = 1/(\alpha + fC)$ where C is the atropine concentration. Therefore f can be estimated from the difference between the values of $1/\tau_{noise}$ in the presence and absence of atropine. Our results indicate that f increases with hyperpolarization, and an e-fold change in f was calculated for a potential change of 150–350 mV. The voltage sensitivity of f is then half that of τ_{noise} in the absence of atropine. This result then leads to the prediction that a reverse voltage sensitivity of τ_{noise} should be expressed at the highest atropine concentrations. We were unable in our experiments at room temperature to measure the effect of high concentrations of atropine on τ_{noise} because of the technical difficulties encountered in measuring cut-off frequencies above 300 Hz. In principle, a similar 'inverse' voltage sensitivity can be expected for $\tau_{e.p.c.}$ and $\tau_{m.e.p.c.}$, which, contrary to τ_{noise}, can be followed in high concentrations of atropine. In most cases, $\tau_{e.p.c.}$ was shortened, with no reversal of voltage sensitivity. Nevertheless a slight tendency to a reverse voltage sensitivity has been detected on m.e.p.c.s in presence of 5×10^{-5} M atropine, and on e.p.c.s. at a concentration of 10^{-4} M (cf. Fig. 2, see also Adler & Albuquerque, 1976, Fig. 7).

One may wonder if, at the higher atropine concentrations, the mean life time of a channel is shortened sufficiently so that the decay of the e.p.c.s is governed by a rate limiting process other than the channel closing, e.g. the rate of fall of the ACh concentration in the synaptic cleft. In fact, the observations of Katz & Miledi (1973*b*) have indicated a fivefold to sevenfold reduction of τ_{noise} for 5×10^{-4} M atropine, whereas at this concentration our results and those of Adler & Albuquerque (1976) give a threefold reduction of $\tau_{e.p.c.}$ If this difference were to be maintained between the two estimates when performed on a single fibre, it would mean that at this concentration the e.p.c. decay is affected by the rate at which the ACh concentration in the cleft declines, a process which is not expected to be voltage dependent. The time course of the e.p.c. in the presence of high doses of atropine (10^{-4} M) does actually fit with the time course of the ACh concentrations evaluated by Magleby & Stevens (1972*a*,*b*) and Beam (1976*b*), that is about 0·3–0·5 ms.

The voltage sensitivity of f could arise from a voltage dependence of atropine binding, but it could also reflect the voltage sensitivity of a conformational change following the binding. This second proposal would make it easier to understand how non-ionized compounds can mimic atropine. For example, Katz & Miledi (1973*b*) have observed

that ether shortens the time course of ACh noise, just as does atropine; octanol makes the decay of m.e.p.c.s faster (Gage, McBurney & Van Helden, 1974) and we have observed in addition that it reduces the voltage sensitivity of the decay phase of the m.e.p.c.s and Adler & Albuquerque (1976) made similar observations on e.p.c.s.

Elementary and total conductance

Like Anderson & Stevens (1973), we have found no voltage sensitivity of γ and the absolute values of γ we observed in normal conditions at low temperature (5–7° C: 20 pS) are consistent with the results of other authors like Colqhoun, Dionne, Steinbach & Stevens (1975) (25 pS), Katz & Miledi (1975*a*) (13 pS), Neher & Sakmann (1975) (23 pS), Dreyer *et al.* (1976) (18 pS).

It is significant that the characteristics of γ are maintained in the presence of atropine. If γ is affected neither by voltage nor by atropine, it is necessary to explain why atropine reduces the conductance at the peak of an e.p.c., an effect which becomes more pronounced at hyperpolarized membrane potential. A presynaptic effect can be ruled out as atropine is known not to affect the quantum content (Beránek & Vyskočil, 1967).

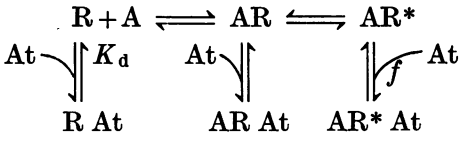
We think that the exclusive binding of atropine to the activated ACh-R complex, as proposed above, is not sufficient to explain this phenomenon and that another binding occurring *before* the channel is open, is necessary to explain our results.

It has been shown (Dionne & Stevens, 1975) that the peak conductance of an ACh response will be proportional to τ_{noise} only when the time ACh is present in the cleft is long relative to the life time of an open channel (e.g. in the case of a steady application of ACh).

In the presence of atropine, τ and $g_{e.p.c.}$ are reduced by about the same extent (in the presence of 5×10^{-5} M atropine, the reduction is about 50%), but τ_{noise} (0·5 ms in these conditions) remains twice the time to peak ACh concentration (0·2–0·3 ms, Magleby & Stevens, 1972*b*, Fig. 4). It would be useful to calculate, in these conditions, to what extent the reduction of τ may affect $g_{e.p.c.}$ But we think that it is already possible to predict that under these conditions the reduction of $g_{e.p.c.}$ should be proportionally smaller than that of τ_{noise}. Therefore it is necessary to suppose that in the presence of atropine, the probability of the channel opening is also reduced: and thus that atropine also binds to the ACh receptor, before the channel is opened. This effect would be increased by hyperpolarization.

Atropine binding sites

Following most authors, we will dissociate two steps in the ACh effect: the ACh A forms an intermediate complex with the receptor R which is then transformed to an activated complex (channel opened). Therefore the bindings of atropine can be written in the following manner



In this scheme, it is emphasized that the effect of atropine on τ can well be explained by the reaction on the right whereas its effect on $g_{e.p.c.}$ needs, in addition, at least one of the two other reactions. From our experiments, some of the constants can be specified. If, for simplicity, the central reaction is omitted, K_d (evaluated from the curve giving the $_{e.p.c.}$ amplitude *vs.* atropine concentration) is around 6×10^{-5} M, and f is in the order of 10^7 M^{-1} s^{-1}, at -90 mV and 20–22° C. In addition, in this scheme, it is predicted that, as soon as b is not nil, a slow tail should appear in the e.p.c. (prediction compatible with our results), and a hump should appear in the low frequency range of the noise spectrum. As already mentioned this component was, because of its small amplitude and slow time course, beyond our technical limits.

One can compare the atropine binding sites to the ACh binding site. On the muscarinic receptor atropine acts as a competitive antagonist for ACh ($K_d = 2 \times 10^{-9}$ M). But this is not the case for the nicotinic receptor, which atropine does not protect from bungarotoxin binding (Lapa, Albuquerque & Daly, 1974). Therefore, for the reactions on the left side of the above model, and by the definition of uncompetitive binding for the reactions on the central and right side of the model the atropine and acetylcholine binding sites must be different.

Many points remain unsolved: are the two binding sites we have defined for atropine by electrophysiological method actually different at the molecular level? Is it still possible for ACh to bind to the atropine-receptor complex? In the case of an affirmative answer to these two questions, one would then have to replace the proposed model by a cyclic model of the kind proposed by Katz & Thesleff (1957) for desensitization or by Changeux *et al.* (1975) for the effect of local anaesthetics.

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