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A TRANSITION TEMPERATURE FOR ACETYLCHOLINE CHANNEL CONDUCTANCE IN CHICK MYOBALLS

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SUMMARY

1. The temperature dependence of ACh channel conductance (γ) and channel open time (τ) was determined by analysing ACh induced membrane current fluctuations in voltage clamped chick myoballs.

2. γ decreased from 25–30 pmho at 37 °C to less than 5 phmo at 10 °C. An Arrhenius plot of γ *vs.* temperature exhibited a clear break or 'transition temperature' at 20 °C.

3. τ increased from 2 msec at 37 °C to 16 msec at 10 °C. The Arrhenius plot of τ *vs.* temperature was linear. No transition temperature was detected.

4. Submicellar concentrations of the non-ionic detergent, Triton X-100 reversibly blocked ACh responses. The effect was all-or-none at the molecular level.

5. These results are consistent with the possibility that the fluidity of membrane lipids in the ACh receptor micro-environment may influence the degree to which the channel can open.

INTRODUCTION

The acetylcholine (ACh) receptor is an integral membrane protein (see Cohen & Changeux, 1975 for review) that must span the lipid bilayer (Katz & Miledi, 1972). Some part of the receptor–ionophore protein complex must therefore be intimately associated with surrounding membrane lipids and it is possible that receptor function is influenced by its hydrophobic micro-environment. In fact, an influence of membrane 'fluidity' has been invoked to explain effects of certain aliphatic alcohols and general anaesthetics on ACh channel kinetics (Gage, McBurney & Schneider, 1975; Gage & Hamill, 1976).

In other systems, breaks in Arrhenius plots of various membrane functions have been taken as *prima facie* evidence for phase transitions or lateral phase separations of membrane phospholipids (see for example Linden, Wright, McConnell & Fox, 1973; Overath & Trauble, 1973; Horowitz, Hatten & Burger, 1974; Kimelberg & Popahadjopoulos, 1974; Houslay, Warren, Birdsall & Metcalfe, 1975). We have used voltage clamped chick myoballs (Fischbach & Lass, 1978) to estimate the ACh channel conductance and mean open time over a wide range in temperature (5°–37 °C). Some of the results have appeared in brief form (Lass & Fischbach, 1976).

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There was a clear break in the Arrhenius plot of channel conductance at about 20 °C, but the plot of channel open time was linear. Similar breaks have been described recently in studies of ACh channel conductance in mouse muscle (Dreyer, Muller, Peper & Sterz, 1976) and of glutamate receptor conductance in locust muscle (Anderson, Cull-Candy & Miledi, 1977).

METHODS

Cell culture and electrophysiological techniques and the analysis of ACh induced current fluctuations were described in the preceding paper (Fischbach & Lass, 1978). Briefly, spherical multinucleated myoballs were prepared by allowing mononucleated precursor cells to fuse in suspension culture. The cells were mechanically dissociated from 11-day-old chick embryo pectoral muscles and were maintained in Eagles' Minimum Essential Medium supplemented

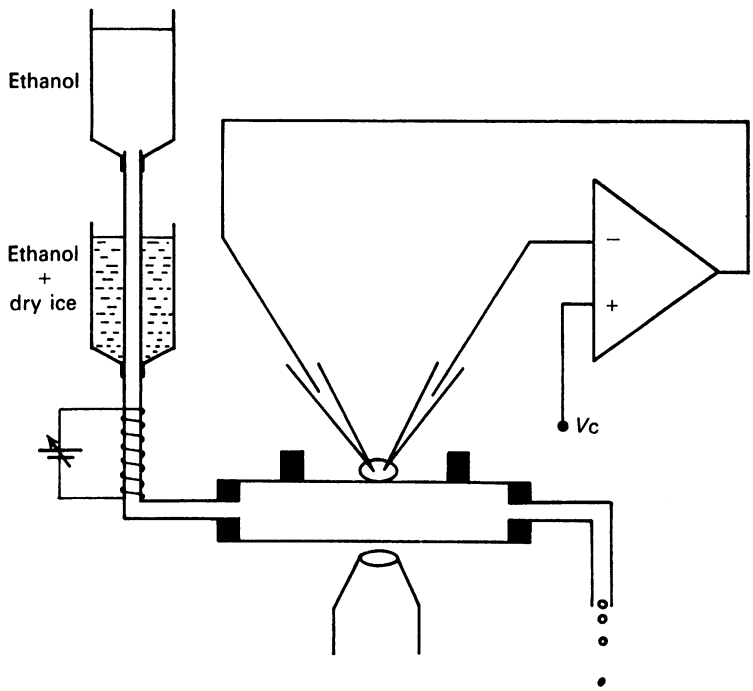

Fig. 1. A diagram of the temperature control apparatus (see text). The ethanol that flowed between the glass plates of the double bottom chamber was first cooled to −30 °C and then warmed to the desired temperature by a heating coil wrapped around the inflow tube. An air jet was directed at the objective to prevent fogging of the lens.

with horse serum (10 % v/v) and chick embryo extract (2 % v/v). ACh currents were measured in voltage clamped myoballs. The single channel conductance (γ) was estimated from the linear relation between the mean ACh conductance ($\bar{G}$) and variance (σ_G^2) of the conductance fluctuations: $\gamma = \sigma_G^2/\bar{G}$. The mean channel open time (τ) was estimated from the power spectrum of the ACh induced fluctuations: $\tau = 1/2\pi f_c$, where f_c is the frequency at which the power is reduced by one half.

Collagen-coated cover-slips (18 mm) with attached myboalls were placed in a double bottomed recording chamber (Fig. 1). The temperature of the medium bathing the cells was changed between 37° and 5 °C by perfusing warmed or cooled ethanol between two glass plates that formed the false bottom of the chamber. The cells were viewed from below with a long

working distance, phase contrast objective. The ethanol was first cooled to −30 °C by a jacket containing dry ice in ethanol and the final temperature was adjusted by a nichrome heating wire wrapped around the inflow tube distal to the cooling jacket. The large temperature gradient between the circulating ethanol and the bath allowed the cells to be cooled from 37 °C to *ca.* 0 °C in 2–4 minutes and during this rapid change the temperature, sensed by a probe placed directly in the bathing medium, varied by less than 2 °C throughout the recording chamber.

In some experiments, Triton X-100 (New England Nuclear) was added to the bathing medium at final concentrations of 0·001 % to 0·01 % (v/v). Detergents bind to serum proteins so the Triton was dissolved in Earle's Balanced Salt Solution which was also used to wash the cells prior to exposure. The concentrations employed were below the critical micellar concentration of Triton (0·015 %, assuming a mean molecular weight of 600; Helenius & Simons, 1975).

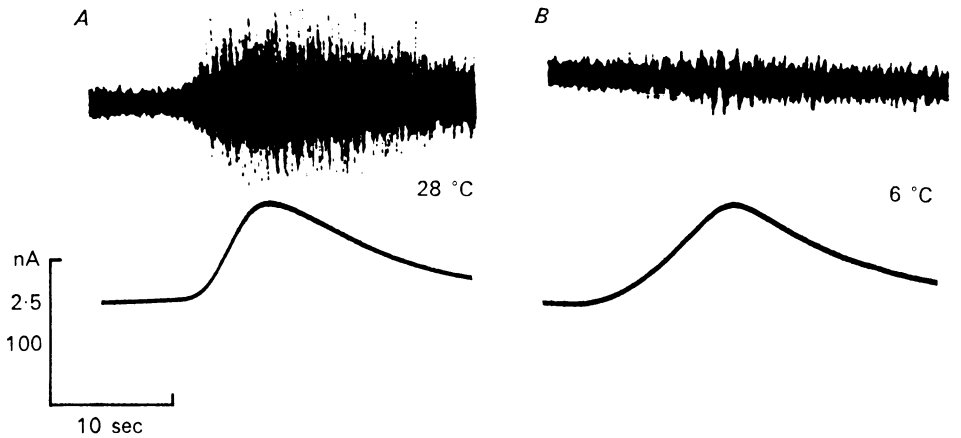

Fig. 2. ACh current noise recorded at 28 °C (*A*) and at 6 °C (*B*) in the same myoball. The lower traces in each pair are low-gain, DC recordings of the mean inward current. The upper traces are high-gain band pass filtered, recordings of the current fluctuations. The vertical bar indicates 2·5 nA for the upper and 100 nA for the lower traces. The slow time course of the responses is determined by diffusion of ACh from a pipette located *ca.* 100 μm away. Note the decrease in the fluctuations at the same mean current recorded at the lower temperature.

RESULTS

Fig. 2 shows ACh responses recorded at 28 °C and at 6 °C in the same voltage-clamped myoball. The lower trace in each pair is a low gain, DC recording of the mean membrane current. The upper traces are high gain, filtered (bandpass 1–250 Hz) recordings that show the current fluctuations. At the lower temperature, there is a dramatic reduction in the current fluctuations at all levels of inward current. The same phenomenon is shown on a faster time scale in the digitalized records of Fig. 3. In this experiment, the single channel conductance (γ) was reduced from 31 pmho at 28 °C to 2 pmho at 6 °C. A comparable decrease was observed in thirty-three additional experiments.

Fig. 4 shows estimates of γ obtained in one myoball at nine temperatures between 37° and 7 °C. There was no significant change in γ as the temperature was lowered from 37° to *ca.* 20 °C, but when the temperature was lowered further to 7 °C, γ decreased fifteenfold. An Arrhenius plot (ln γ *vs.* $1/T$) of data from seven myoballs (normalized to the same γ at 37 °C) is shown in Fig. 5. Despite the pooling of data,

an abrupt change in slope at *ca.* 20 °C is evident. The activation energy (E_a) was calculated from the relation

$$\ln \gamma = A - \frac{E_a}{2{\cdot}3R}\left(\frac{1}{T}\right), \tag{1}$$

where R is the gas constant, T is the temperature in degrees Kelvin and A is a constant. At the transition temperature of 20 °C, the E_a for those events which determine the degree to which a channel can open increased by 27 kcal/mole.

Fig. 3. Digitalized records of 'background' noise (upper trace) and of ACh induced noise at 23 °C (middle trace) and 9 °C (lower trace). The mean current required to clamp the membrane at −70 mV is defined as 0 nA. A PDP8e computer was used to sample the noise and for subsequent calculations.

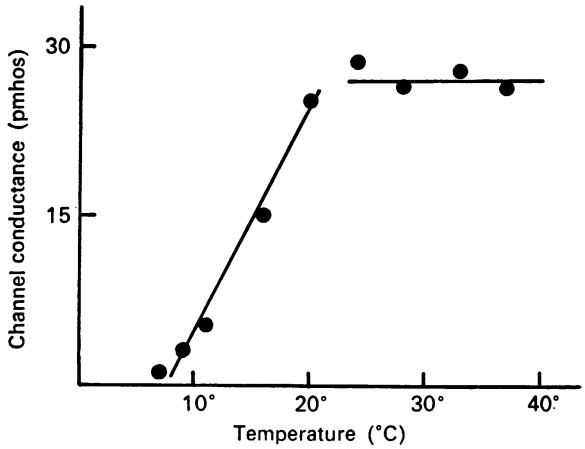

Fig. 4. The relation between single channel conductance and temperature between 5° and 37 °C. Estimates of γ are based on nine ACh responses evoked in the same myoball at various temperatures.

The current that flows through an activated channel depends on the driving force ($V_m - V_{ACh}$) as well as the channel conductance. Thus, a negative shift in the ACh reversal potential (V_{ACh}) could account for a reduction in the measured current

fluctuations. But as shown in Fig. 6, this was not the case. The membrane potential (V_m) of a myoball examined at 5 °C was changed by a ramp command voltage and several ACh responses were elicited during the slowly rising (1 mV/sec) ramp. V_{ACh}, determined by interpolation, was −5 mV. The same result was obtained in four other cells, and we conclude that V_{ACh} is the same at 5 °C as at 37 °C (Fischbach & Lass, 1978).

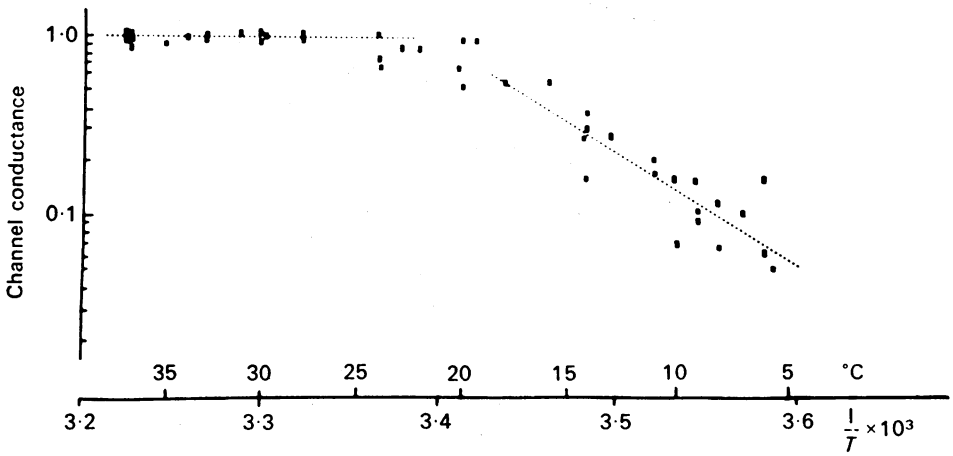

Fig. 5. An Arrhenius plot of single channel conductance including data from seven myoballs. In each case, the data were normalized by setting γ estimated near 37 °C equal to 1·0. C = degrees Centigrade; T = degrees Kelvin. Note the abrupt change in slope at *ca.* 20 °C.

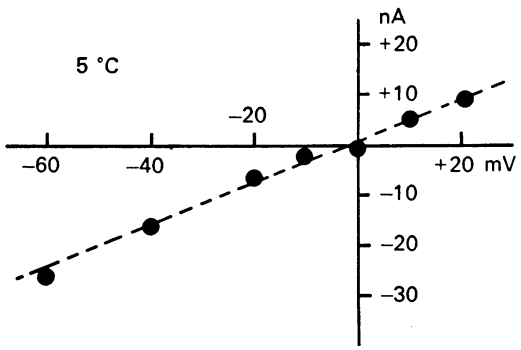

Fig. 6. The ACh reversal potential at 5 °C. The membrane potential was changed by a slowly rising ramp command voltage (1 mV/sec) and several identical ACh pulses were delivered during the ramp. The points measure the peak ACh currents. Inward current to the left and outward current to the right of the ordinate.

In addition to the decrease in the amplitude of the noise, the digitalized records in Fig. 3 show that relatively low frequency components of the ACh induced fluctuations predominate at 9 °C. Thus, it is possible that the amplitude of the fluctuations was reduced at low temperature simply because they were attenuated by the band pass filter. However, the records shown in Fig. 7 indicate that this is unlikely; ACh responses evoked in the same myoball at 5 °C were recorded at the usual filter settings of 1–250 Hz (A) and then with the low cut-off frequency reduced

to 0·1 Hz (*B*). The noise-to-noise ratio was low in both cases but no increase in the amplitude of the fluctuations was observed at the lower cut-off frequency. The triphasic wave in *B* is an artifact caused by the large DC component in the signal.

At 5 °C the resistance of the recording electrode increased about twofold. In theory, the r.m.s. value of the feed-back current should *increase* by about 0·1 nA within the frequency rate of interest. This, obviously, cannot account for the large

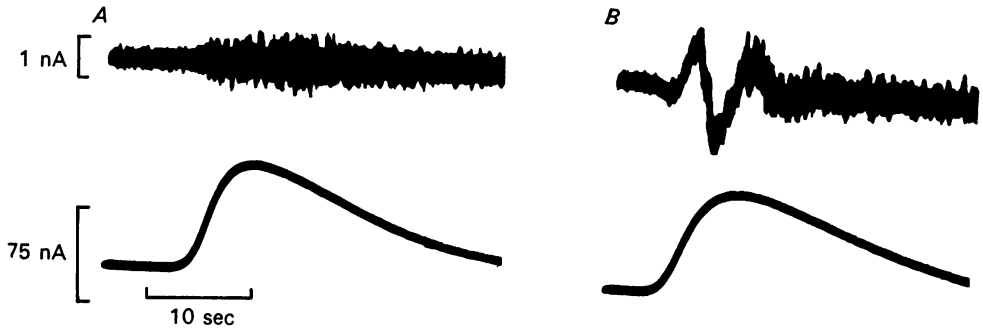

Fig. 7. Two ACh responses recorded in the same myoball at 7 °C. In *A*, the band pass filter was set at 1–250 Hz and in *B* it was set at 0·1–250 Hz. The maximum amplitude of the noise was the same at both settings. The triphasic wave is an artifact.

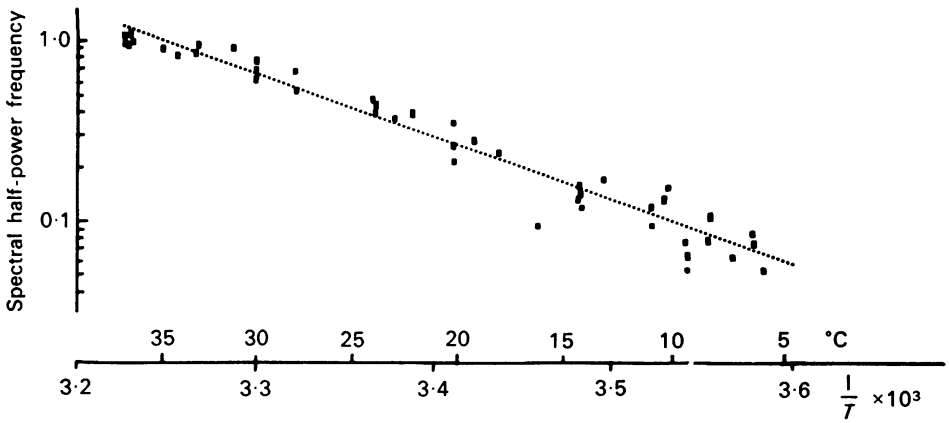

Fig. 8. An Arrhenius plot of half-power frequency ($f_c = 1/2\pi\tau$). No change in slope is evident over the entire temperature range investigated.

decrease in ACh fluctuations observed at low temperature. As the temperature was decreased the tip of the ACh pipette often bent away from the myoball. This resulted in a slower (diffusion limited) ACh current (cf. Fig. 2), but as all ACh pulses were within the 'low ACh concentration' range (Fischbach & Lass, 1978) this did not alter our estimates of γ. In most cases, the size of the pulse was increased or the electrode was re-positioned to achieve a constant peak current.

As stated above, low frequency components of ACh fluctuations predominated at low temperature. Power spectra were shifted to the left along the frequency axis. In the experiment shown in Fig. 3, the mean receptor open time (τ), estimated from the half-power frequency (f_c), increased from 7·2 msec at 23 °C to 16·1 msec

at 9 °C. An Arrhenius plot of the data pooled from seven myoballs (normalized to the same f_c at 37 °C) is shown in Fig. 8. No obvious break in the relation was noted, and the data were best fit by a single straight line. The activation energy for those events which determine the rate of channel closing was 16 kcal/mole.

In eighteen experiments, low concentrations of Triton X-100 reduced or eliminated the ACh induced depolarization of myoballs without affecting membrane potential or resting conductance. A typical experiment in which pulses of ACh were delivered at a rate of 0·5/min is plotted in Fig. 9. In most cases, the block was rapidly reversible.

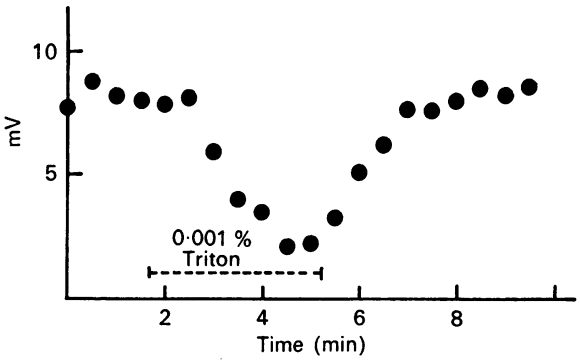

Fig. 9. The reversible block of ACh responses by Triton X-100. This myoball was not voltage clamped. The peak amplitude of successive responses (depolarizations) to identical pulses of ACh delivered at 0·5/min is plotted *vs.* time. Triton (0·001 %) was included in the bathing medium during the period shown by the dashed line. The long latency reflects the slow rate of perfusion.

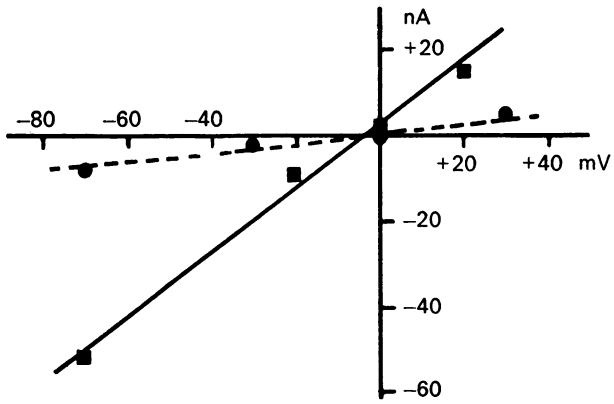

Fig. 10. The ACh reversal potential in control salt solution (squares) and in the presence of 0·005 % Triton X-100 (circles). The membrane potential was changed by a slowly rising ramp command voltage (1 mV/sec). The points are peak currents in response to identical pulses of ACh. Although Triton reduced the membrane currents at each membrane potential, there was no change in the ACh reversal potential (Y-intercept).

This inhibition was not due to a negative shift in the ACh reversal potential. Fig. 10 shows voltage–current curves obtained during the action of ACh in the presence (circles) and absence (squares) of 0·005 % Triton. Although the ACh

induced current was markedly diminished at all potentials, the interpolated reversal potential was unchanged. Analysis of ACh induced membrane current fluctuations in the presence of concentrations of Triton that reduced the mean ACh current by more than 50 % failed to reveal any change in single channel conductance or mean channel open time. This result was obtained in five different cells.

DISCUSSION

One interpretation of an abrupt change in slope in the relation between ACh channel conductance and temperature is that the degree to which an activated channel can open is influenced by the fluidity of surrounding membrane lipids. Evidence for this view comes from studies of bacterial membranes where breaks in Arrhenius plots of membrane transport have been correlated with transition temperatures of extracted membrane phospholipids (Overath & Trauble, 1973). A variety of physical techniques indicate that above the critical temperature unsaturated fatty acid side chains are flexible, whereas below this temperature, lateral and/or rotational motion is restricted.

While this argument by analogy is appealing, other possibilities must be borne in mind. For example, the receptor-ionophore protein complex itself may undergo a conformational change at the critical temperature. Alternatively, the conductance of an open channel may be governed by a sequence of reactions and one may become rate limiting at 20 °C. Whatever the precise mechanism, it must account for the fact that the marked decrease in channel conductance at low temperature is not associated with a change in ion selectivity.

It is significant that below 20 °C the channel conductance decreased gradually over a rather broad range in temperature (20°–5 °C). This cannot be explained simply on the basis of heterogeneity of membrane lipids. If channels in different lipid microenvironments were 'frozen' at different temperatures, no change in the conductance of single channels would be observed. Rather, the fivefold graded decline in channel conductance implies a continuum of open states. The possibility of several open configurations is also supported by small differences in channel conductance observed with different nicotinic agonists (Colquhoun, Dionne, Steinbach & Stevens, 1975). Direct recordings of single channel events (cf. Neher & Sakmann, 1976) at different temperatures are needed.

It is noteworthy that although the mean channel open time was prolonged with decrease in temperature, between 37° and 5 °C no obvious break in the Arrenhuis plot was observed. This finding has been confirmed by analysis of extracellularly recorded ACh noise in elongated chick myotubes (S. Schuetze and G. D. Fischbach, unpublished data). Similarly in the mouse, where an apparent transition temperature for the ACh channel conductance was observed at *ca.* 20 °C, the Arrhenius plot of channel open time *vs.* temperature was linear between 37° and 10 °C (Dreyer *et al.* 1976). A linear Arrhenius plot is not, of course, strong evidence against an influence of membrane fluidity on channel kinetics (cf. Gage *et al.* 1975; Gage & Hamill, 1976) However, as a limiting statement, it seems clear that the equilibrium conformation of the activated ACh receptor can be altered without altering the kinetics of the conformational change. Those phenomena which regulate the degree to which the

channel can open must be different from those which regulate the rate of channel closing. The same conclusion can be drawn from studies of amphibian ACh receptors. There is little, if any, change in channel conductance between 22° and 10 °C but the channel open time increases three to fourfold over the same range (Anderson & Stevens, 1973). Glutamate receptors in locust muscle are different. A break in the Arrhenius plot of channel open time occurs at the same temperature (6 °C) as the break in the channel conductance *vs.* temperature relation (Anderson *et al.* 1977).

Our results with Triton X-100 confirm previous reports that low concentrations of this detergent block nicotinic ACh responses (Bartels & Rosenberg, 1972; Fambrough, 1974; Changeux, Benedetti, Bourgeas, Brisson, Cartoud, Devaux, Grunhagen, Moreau, Popet, Sobel & Weber, 1975). In addition, we found that the block was not associated with a change in membrane potential or input resistance. Nor was there a change in ACh channel conductance or ion selectivity or in mean channel open time. In contrast to other agents that enter the membrane lipid bilayer (and alter ACh channel kinetics), such as long chain alcohols, local anaesthetics and general anaesthetics (Katz & Miledi, 1975; Gage *et al.* 1975; Gage & Hamill, 1976), Triton apparently eliminates receptors in an all-or-nothing fashion.

Considering that Triton was effective at 0·001 %, it is extremely unlikely that the reduced ACh response is due to the loss of receptors from the membrane. In fact, in two experiments, we could detect no decrease in binding of [^{125}I]α-bungarotoxin to Triton treated myoballs (cf. Fambrough, 1974; Changeux *et al.* 1975). The efficacy of Triton is consistent with the notion that ACh channel function is modulated by membrane lipids, but other possibilities remain. Triton monomers bind tightly to various proteins (Helenius & Simons, 1975) and they may induce or modify conformational changes directly. It should be emphasized that the all-or-none action of Triton (on channel conductance) is quite different from the graded effect of lowering temperature below 20 °C. Thus, these treatments probably do not exert their effects in exactly the same way.

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