

METHODS

Experiments were made on sartorius muscles of the frog (*Rana temporaria*) maintained at 4–6 °C. Endplates were located visually under the compound microscope (Katz & Miledi 1965) and were voltage-clamped with two microelectrodes, while ACh was applied iontophoretically from another, external micropipette. Control Ringer solution contained NaCl, 115 mM; KCl, 2 mM, CaCl₂, 1.8 mM; HEPES buffer, 4 mM; at pH 7.2. Isotonic sucrose solution contained sucrose, 240 mM; K⁺, 2 mM; HEPES, 4 mM; at pH 7.2. KOH was used to set the pH of the sucrose solution and this is included in calculating the K⁺ concentration. Muscles were rinsed several times with isotonic sucrose solution before measurements were taken.

A fast Fourier transform method was used to analyse the membrane current fluctuations seen during application of ACh, and the elementary current and mean lifetime of the postsynaptic channels induced by ACh were determined as previously described (Katz & Miledi 1972; Anderson & Stevens 1973).

RESULTS

Muscle survival in isotonic sucrose

In experiments performed several years ago it was noticed that, while muscles did not survive well in isotonic sucrose at room temperature, they remained in good condition for many hours when kept at low temperatures. In view of this the present experiments were conducted at 4–6 °C. In these conditions the resting potential and input resistance of the muscle fibres remained high for several hours. For instance in one set of muscles in isotonic sucrose the resting potential was -78.3 ± 7 mV (mean \pm s.d., 15 fibres) and the input resistance measured from -90 to -110 mV was about 400 k Ω . These values are probably artificially low due to membrane damage caused by the relatively large microelectrodes that we used to improve the performance of the clamp. The muscle fibre membrane was surprisingly robust in isotonic sucrose and it was possible to clamp it over a wide range of potentials. The experiments were also facilitated by the absence of contraction when the membrane was depolarized. This abolition of contraction appears to be due, at least partly, to a shift in the threshold for inactivation of the depolarization–contraction coupling mechanism (unpublished results).

ACh-induced currents in low ionic strength media

It was immediately obvious that the ACh receptor–channel system was still functioning after prolonged incubation in isotonic sucrose because miniature endplate currents (m.e.p.c.), due to the release of packets of ACh from motor nerve terminals, were easily detected. The m.e.p.c. frequency increased approximately 200-fold soon after changing the normal bathing solution to isotonic sucrose, and sometimes was so high that it was difficult to distinguish individual

events. After several hours in isotonic sucrose, most, if not all, endplates were still sensitive to ACh released from nerve terminals or from an iontophoretic pipette.

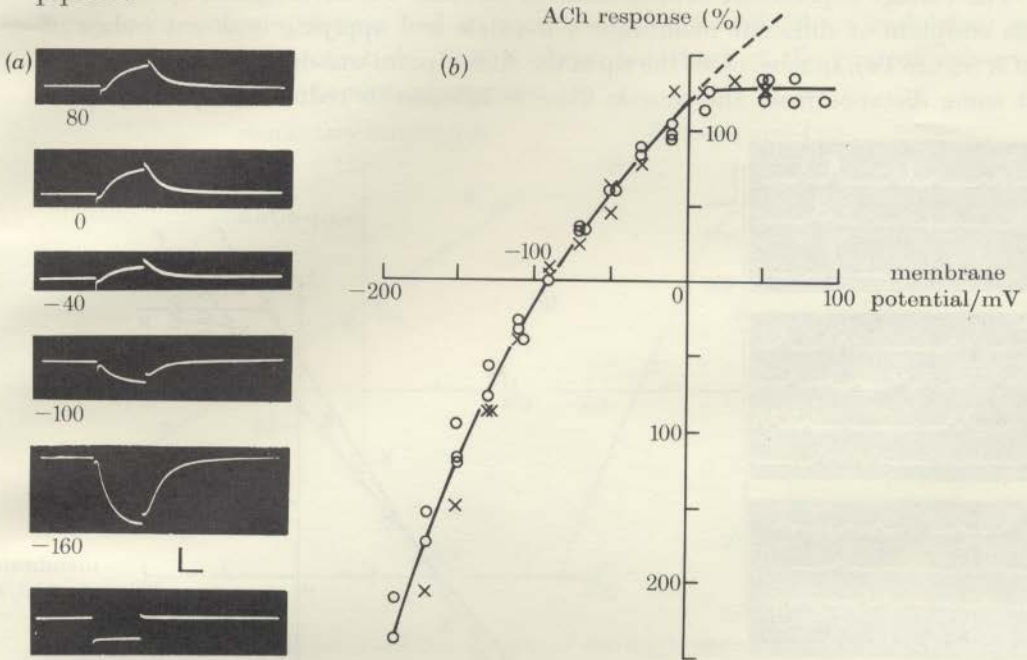


FIGURE 1. Endplate currents elicited by iontophoretic pulses of ACh in isotonic sucrose solution. (a) Records of ACh responses at different membrane potentials; numbers give potentials in millivolts. The lowest trace shows the iontophoretic current through the ACh pipette. Due to the low conductivity of the sucrose solution a fraction of the iontophoretic current was picked up on the clamp current monitor, and this has been subtracted from all measurements. Calibration bars: horizontal 500 ms; vertical 10 nA for both clamp and iontophoretic current. Same endplate as for figure 3b. Temperature 5.2 °C. (b) Voltage-current relation for the ACh-induced response. Data are shown for two endplates from different muscles. The results have been normalized by expressing them as a percentage of the response at a potential 50 mV hyperpolarized from equilibrium for each fibre; 100% corresponds to currents of 9 nA (x) and 12 nA (o). Curves were fitted by eye.

The equilibrium potential of ACh action was determined directly from the reversal potential of ACh currents induced by iontophoretic pulses of ACh. The mean value from 21 endplates in isotonic sucrose plus 2 mM K^+ was -85 ± 10.6 mV and a value of -11 mV was recorded from one endplate in normal Ringer. Thus the equilibrium potential for ACh action in isotonic sucrose is very close to the resting potential and it was usually necessary to displace the membrane potential in either direction, to see clearly m.e.p.cs or iontophoretically induced ACh currents. An experiment with different K^+ concentrations in the sucrose solution showed that the equilibrium potential varied as the logarithm of K^+ concentration, with a slope close to that of the Nernst equation (55 mV per concentration

decade). It seems, therefore, that in the sucrose solution the major part of the endplate current is carried by K^+ ions.

The voltage dependence of ACh-induced currents was investigated by clamping the endplate at different membrane potentials and applying constant pulses of ACh (figure 1*a*). In most cases the tip of the ACh pipette was deliberately positioned at some distance from the muscle fibre membrane to reduce the possibility of

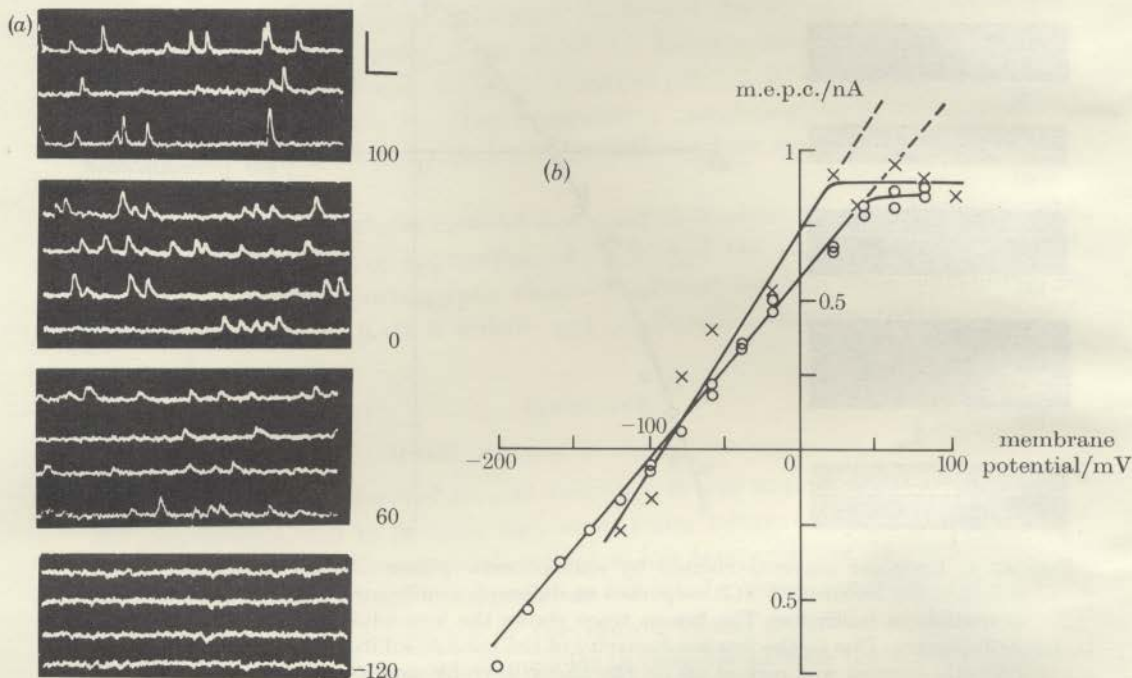


FIGURE 2. (*a*) M.e.p.c.s recorded at different membrane potentials (given in millivolts) in sucrose solution. Records are unselected. Calibration bars: horizontal, 20 ms; vertical 2 nA. Currents were filtered at 500 Hz, and a.c.-coupled with a time constant of 0.1 s. Temperature 4 °C. (*b*) Voltage-current relation of peak m.e.p.c. amplitude in isotonic sucrose solution. Data from two endplates are shown. Points \times are the same fibre as for (*a*). Lines were fitted by eye.

artefacts caused by spurious changes in the distance between source of ACh and receptors in the muscle membrane. An almost linear relation between amplitude of ACh current and potential was observed over the range of about -200 to near 0 mV, but thereafter a striking departure was seen, with the current amplitude remaining steady (figure 1*b*), or even decreasing (figure 3*b*), as the potential was shifted to more positive values.

M.e.p.c. and single channel currents

In the experiments illustrated in figures 1 and 3, the duration of the pulses of ACh was relatively long. However, a similar voltage-ACh current relation is

observed when much shorter pulses of ACh are used. Moreover, the same type of relation pertains for the amplitude of m.e.p.c. (figure 2*b*) caused by the very brief jets of ACh released from the nerve terminals. The peak conductance during the m.e.p.cs in isotonic sucrose had a mean value of 8.4 nS (three endplates) at negative membrane potentials. This is about one-third of the control value in normal Ringer, at the same temperature.

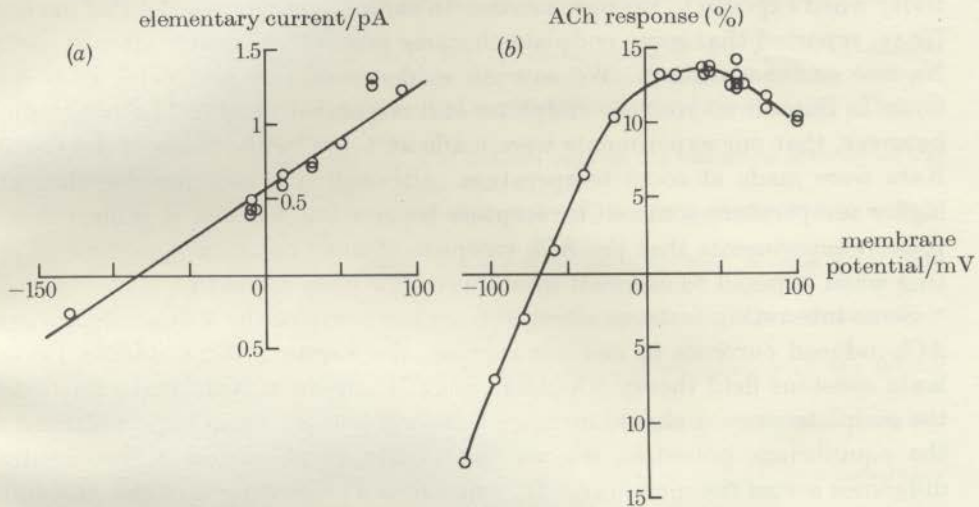


FIGURE 3. (a) Membrane potential dependence of the single channel current measured from one endplate in isotonic sucrose solution. The elementary currents were estimated from the total spectrum variance after subtraction of background variance and have been corrected for loss of high frequencies due to filtering (Colquhoun *et al.* 1977). Each point is a value from one noise run, during which about 20 segments of 512 points were digitized at a rate of 1 kHz. To remove noise contributions caused by high frequency m.e.p.cs the experiment was performed on a muscle that had been denervated 6 days previously. Temperature 5–6 °C. The line was fitted by eye. (b) Voltage–current relation for the ACh-induced current recorded from the same endplate as for (a). Responses were elicited by 1 s duration ACh pulses. The curve was fitted by eye.

Miniature endplate currents result from the nearly simultaneous opening of many membrane channels. To see if the voltage dependence of single channel currents was similar to that for pulses of ACh or m.e.p.c. we used ACh noise analysis to estimate single channel characteristics in isotonic sucrose. In contrast to currents induced by iontophoretic pulses of ACh, or by the packages of ACh released from the nerve terminals, the amplitude of the elementary current varied linearly with membrane potential at all voltages examined (figure 3*a*). The mean single channel conductance measured at four endplates was 4.1 pS compared to a control value of 18.3 pS obtained in four endplates from the same muscles bathed in normal Ringer. The voltage dependence of the channel lifetime in isotonic sucrose was reduced but was still in the same direction as seen in normal Ringer. An e-fold change in lifetime was produced by a potential shift

of about 200 mV (compared with about 60 mV in normal Ringer), and no change in the voltage dependence of the lifetime was apparent at positive membrane potentials.

DISCUSSION

Our experiments show that the muscle fibre membrane retains its ACh sensitivity when exposed to isotonic sucrose. In earlier experiments, del Castillo & Katz (1955) reported that some endplates became insensitive to ACh after exposure to Na-free sucrose medium. We saw no evidence of this and even after several hours in isotonic sucrose the endplates still responded to ACh. It should be noted, however, that our experiments were made at 4–6 °C while those of del Castillo & Katz were made at room temperature. Although it is still possible that at the higher temperature some ACh receptors become inactivated, it is clear from the present experiments that the ACh receptor-channel complex is capable of operating when exposed to external solutions of low ionic strength.

Some interesting features emerged from the study of the voltage dependence of ACh-induced currents in isotonic sucrose. For instance the Goldman-Hodgkin-Katz constant field theory (Goldman 1943; Hodgkin & Katz 1949) predicts that the endplate current should increase less than linearly when hyperpolarized from the equilibrium potential, due to the highly asymmetrical K^+ concentration difference across the membrane. In contrast, our observations of the ACh-induced currents, m.e.p.cs and single channel currents suggest that a linear relation is followed at hyperpolarized potentials.

Perhaps even more interesting is the 'saturation' of K^+ efflux induced by iontophoretic pulses of ACh, or by transmitter quanta, when the membrane was held at positive potentials (figures 1, 2). Several factors could contribute to produce this effect. For example: a limited availability of K^+ ions just inside the membrane or an accumulation of K^+ just outside; an iontophoretic removal of ACh from the vicinity of the ACh receptors; channel plugging by an internal cation; a decrease in the conductance of the single channel. These possibilities will be discussed at a later date. It is sufficient to say here that none of them seems likely to play a major role in causing the plateau or decrease of ACh current observed at positive membrane potentials. The fact that the amplitude of the single channel current varied linearly with membrane potential, even when the gross ACh current was clearly decreasing (figure 3), suggests a more attractive possibility. It is as if the number of functional ACh-induced channels decreases as the membrane is driven to positive potentials, but that those that remain function with the lifetime and conductance expected for those potentials. Perhaps the positive electric field across the membrane causes the receptor protein subunits to be displaced in such a way that the ACh is unable to bind to the receptor, or, if it binds, is unable to cause the channel to open.

Endplate currents in sucrose solution

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Endplate currents were recorded from voltage-clamped frog muscle fibres bathed in an isotonic sucrose solution containing 2 mM K^+ . In this solution the major part of the current is carried by K^+ ions, and at negative potentials the membrane voltage-current amplitude relations of both miniature endplate currents and the single channel current estimated from noise analysis were linear, with smaller conductance than in normal Ringer solution. At positive potentials miniature endplate currents and currents induced by acetylcholine (ACh) showed a saturation, or sometimes even decline, with increasing potential. In contrast, the single channel current continued to increase linearly at these potentials. It is suggested that in sucrose solution the number of functional ACh receptors decreases as the endplate is depolarized to more positive potentials.

INTRODUCTION

The synaptic current at the frog endplate is known to be carried principally by Na^+ and K^+ ions (del Castillo & Katz 1955; Takeuchi & Takeuchi 1960), with a smaller contribution from Ca^{2+} ions (Takeuchi 1963; Bregestovski *et al.* 1979). The synaptic current arises because the reaction of acetylcholine (ACh) with receptors embedded in the muscle fibre membrane leads to the transient opening of channels, through which the ions can flow. With use of 'noise' analysis (Katz & Miledi 1970, 1972), it is possible to estimate the lifetime and conductance of these channels, and a good deal is already known about their performance when exposed to media of normal ionic strength. We were interested to study the behaviour of the system in a solution in which all the Na^+ and Ca^{2+} was replaced by sucrose for two reasons: first to examine the effects of low ionic strength on the ACh receptor-channel complex, and secondly to examine the K^+ component of the endplate current in isolation. ACh-induced currents had previously been observed in endplates bathed in Na^+ -free sucrose solution, but these responses were irregular and small (Nastuk 1953; del Castillo & Katz 1955).

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