EFFECTS OF STRONTIUM IONS ON END-PLATE CHANNEL PROPERTIES

By R. MILEDI and I. PARKER

From the Biophysics Department, University College, Gower St., London WC1E 6BT

SUMMARY

1. Changes in end-plate channel properties resulting from substitution of Sr^{2+} for Ca^{2+} in the Ringer solution have been analysed at the voltage clamped frog end-plate, by recording m.e.p.c.s and ACh induced noise variance.

2. In 2 mM-Sr^{2+}-Ringer the peak size of m.e.p.c.s showed a very small increase, and the time constant of the decay phase ($\tau_{m.e.p.c.}$), at any given voltage, was increased by a factor of about two compared to control Ringer. The voltage dependence of $\tau_{m.e.p.c.}$ was the same in both solutions.

3. Addition of increasing amounts of $CaCl_2$ to 2 mM-Sr^{2+}-Ringer produced a progressive shortening of $\tau_{m.e.p.c.}$, with no change in voltage dependence.

4. Estimates of single channel properties from noise analysis showed that the elementary conductance appeared to be slightly increased in 2 mM-Sr^{2+}-Ringer, whilst the mean channel life-time was prolonged by a factor of about two. These changes in single channel properties are sufficient to account for the observed changes in m.e.p.c.s.

5. Following inhibition of cholinesterase activity by neostigmine, similar effects on m.e.p.c.s and single channel properties were still observed on changing to 2 mM-Sr^{2+}-Ringer. The shapes of m.e.p.c.s in Sr^{2+} + neostigmine Ringer were often altered, and showed flat 'plateaus'.

6. The observed effects of Sr^{2+}-Ringer on channel life-time cannot be explained on the basis of changes in surface charge density on the membrane, and suggest that divalent cations have an additional, and more direct, influence on receptor channel properties.

INTRODUCTION

When Ca ions are present in the external medium bathing a muscle, depolarization of presynaptic motor nerve terminals leads to the release of transmitter quanta, which act on the muscle fibre membrane, and produce miniature end-plate potentials (del Castillo & Katz, 1954). In the absence of Ca^{2+}, presynaptic membrane depolarization fails to release transmitter; but if Sr^{2+} is substituted for Ca^{2+}, transmitter is again released in quantal form (Miledi, 1966).

The transmitter quanta released in the presence of Sr^{2+}, evoked miniature end-plate potentials which were larger than those caused by quanta released in the presence of Ca^{2+}. Moreover, it appeared that the larger size of the miniature potentials in Sr^{2+} was due, not to an increase in the number of acetylcholine (ACh) molecules

in the quanta but to an increased effectiveness of ACh on the post-synaptic membrane (Dodge, Miledi & Rahamimoff, 1969). Since the combination of ACh with receptors in the muscle membrane, leads to the opening of membrane channels, with a given life-time and conductance (Katz & Miledi, 1972; Anderson & Stevens, 1973), the increased effectiveness of ACh, in the presence of Sr^{2+}, could be due to an increase of either one or both of these channel characteristics, or to an increase in the number of ACh- receptor collisions leading to channel openings. The present experiments were aimed at exploring these questions.

METHODS

Experiments were performed on the sartorius muscle of *Rana temporaria*, at a temperature of 5–7 °C. The control Ringer solution contained (mM); NaCl 120; KCl 2; $CaCl_2$ 1·8; HEPES 4; at pH 7·2. Strontium Ringer had the same composition, except that 2 mM-Sr Cl_2 was substituted for $CaCl_2$.

End-plates were located visually under a compound microscope (Katz & Miledi 1965), and were voltage clamped using a conventional two point clamp. The gain of the clamp was adjusted so that the residual voltage from miniature end-plate currents (m.e.p.c.s) was undetectable at a gain of 1 mV cm^{-1}. M.e.p.c.s and ACh induced current fluctuations were recorded through a virtual earth current monitor, filtered with a band width of 0·02–500 Hz, and recorded on FM tape. For noise experiments, a micropipette containing 1 M-ACh was used to apply ACh ionophoretically to the end-plate.

The decay time constant of m.e.p.c.s. ($\tau_{m.e.p.c.}$) was analysed by sampling m.e.p.c.s from tape, using a PDP 11 computer, at a digitization rate of 1000 or 500 Hz. Between 5 and 20 m.e.p.c.s from each run were averaged by summation, after visually aligning the peaks. A logarithmic plot of the averaged decay phase was then displayed on a screen, and a straight cursor line was fitted by eye to this. $\tau_{m.e.p.c.}$ (time to fall to $1/e$) was computed from the slope of the line. M.e.p.c.s with very slow rise times, or rounded tops, were excluded from analysis.

Records of current fluctuations during steady local application of ACh were fed via an active filter (100 Hz low pass. $1/f^8$ roll-off) into a PDP11 and digitized at 200 Hz sample rate. Power spectra were computed for 512 point segments of each record using a fast Fourier transform algorithm, an average of 10–20 spectra being formed for each run. Averaged control noise spectra taken immediately before application of ACh were subtracted from noise spectra during drug application. Spectra were analysed as previously described (Anderson & Stevens, 1973; Katz & Miledi, 1972). The mean channel lifetime (τ_{noise}) was estimated by measuring the half-power frequency of the spectra (f_c), and calculating $\tau_{noise} = 1/2(\pi f_c)$.

The mean single channel current was calculated from the total spectrum variance, after subraction of background variance, by dividing by the mean ACh induced end-plate current.

RESULTS

M.e.p.c.s in 2 mM-Sr^{2+}–Ringer solution

Experiments were generally performed by obtaining control records from several fibres whilst bathing the muscle in control Ringer solution, and then obtaining records from the same, and additional fibres, after changing the solution to 2 mM-Sr^{2+}–Ringer. Muscle fibre resting potentials were not appreciably different in the two solutions; mean values, after penetration with both electrodes, were $-85·5 \pm 6·4$ mV (nineteen) in Sr^{2+}–Ringer, and $-85·7 \pm 5·2$ mV (ten) in control solution (figures give mean, standard deviation, and number of fibres). The peak size of m.e.p.c.s showed a very small increase after changing to 2 mM-Sr^{2+}–Ringer, the mean size from all fibres examined (seven muscles) being 106 % (± 11 % standard deviation) of the

control. This effect was examined more carefully in one experiment by measuring m.e.p.c.s from film (to avoid possible loss of small m.e.p.c.s which may have failed to trigger the computer), and including data only from end-plates examined in both solutions. 40 mM-sucrose was added to the bathing solution to increase m.e.p.c. frequency. The peak m.e.p.c. sizes in Sr^{2+}–Ringer varied between 94 and 110% of the control values at different end-plates, and the mean value from five end-plates was 104%.

Large differences were however apparent in the decay times of m.e.p.c.s recorded in the two solutions. This is illustrated in Fig. 1A, which shows averaged m.e.p.c.s recorded from the same end-plate at a potential of -130 mV, in both solutions. At this end-plate the decay time constant $\tau_{m.e.p.c.}$ was 13·5 msec in control solution, and 27·4 msec in Sr^{2+}–Ringer. A similar lengthening of decay time was seen in all fibres examined. In both solutions the m.e.p.c. decay could be fitted adequately by a single exponential, although on the logarithmic display the decay sometimes showed two straight line segments, with slopes differing by up to about 10%.

Fig. 1B shows pooled data from nineteen fibres of the voltage dependence of $\tau_{m.e.p.c.}$ in both solutions. $\tau_{m.e.p.c.}$ is known to vary exponentially with membrane potential in normal Ringer solution (Kordaš, 1969; Magleby & Stevens, 1972) A similar relationship also applies in Sr^{2+}–Ringer, as demonstrated by the close fit of the data to a straight line, on the semilogarithmic co-ordinates of Fig. 1B. The voltage constant (potential shift to give an e-fold change in $\tau_{m.e.p.c.}$ was 54 mV in control solution, and 60 mV in Sr^{2+} solution. At any given potential however, $\tau_{m.e.p.c.}$ was greater by a factor of about 2 in Sr^{2+}–Ringer, which is equivalent to a shift of the relationship along the voltage axis of approximately 45 mV in a hyperpolarizing direction.

Addition of Ca^{2+} to Sr^{2+}–Ringer solution

The prolongation of m.e.p.c. decay times seen in 2 mM-Sr^{2+}–Ringer could be antagonised by addition of Ca^{2+} ions to the solution. This is illustrated in Fig. 2, which shows the voltage dependence of $\tau_{m.e.p.c.}$ at end-plates bathed in Sr^{2+}-Ringer, to which different amounts of $CaCl_2$ were added. Addition of 1 mM-Ca^{2+} caused a shortening of m.e.p.c. decay time to about 0·77 of the value in Sr^{2+}, whilst after addition of a total of 10 mM-Ca^{2+}, $\tau_{m.e.p.c.}$ had decreased close to the values in normal Ringer. The voltage dependence of $\tau_{m.e.p.c.}$ was approximately the same in all solutions, as shown by the closely parallel lines on the semilogarithmic plot of Fig. 2

Noise analysis of single channel properties

Possible explanations for the prolongation of $\tau_{m.e.p.c.}$ in Sr^{2+}-Ringer include; (i) an increase in mean life-time of the elementary end-plate current, and (ii) an inhibitory effect of Sr^{2+} on the cholinesterase, leading to an increase in repetitive binding of ACh to receptors in the synaptic cleft (Katz & Miledi, 1973). To distinguish between these possibilities we studied the single channel properties, by analysing the voltage clamped noise which occurs during steady ionophoretic application of ACh (Katz & Miledi 1972; Anderson & Stevens, 1973).

Fig. 3A shows a typical spectrum of current fluctuations induced by ACh at an end-plate bathed in 2 mM-Sr^{2+} solution. The data could be fitted well by a single
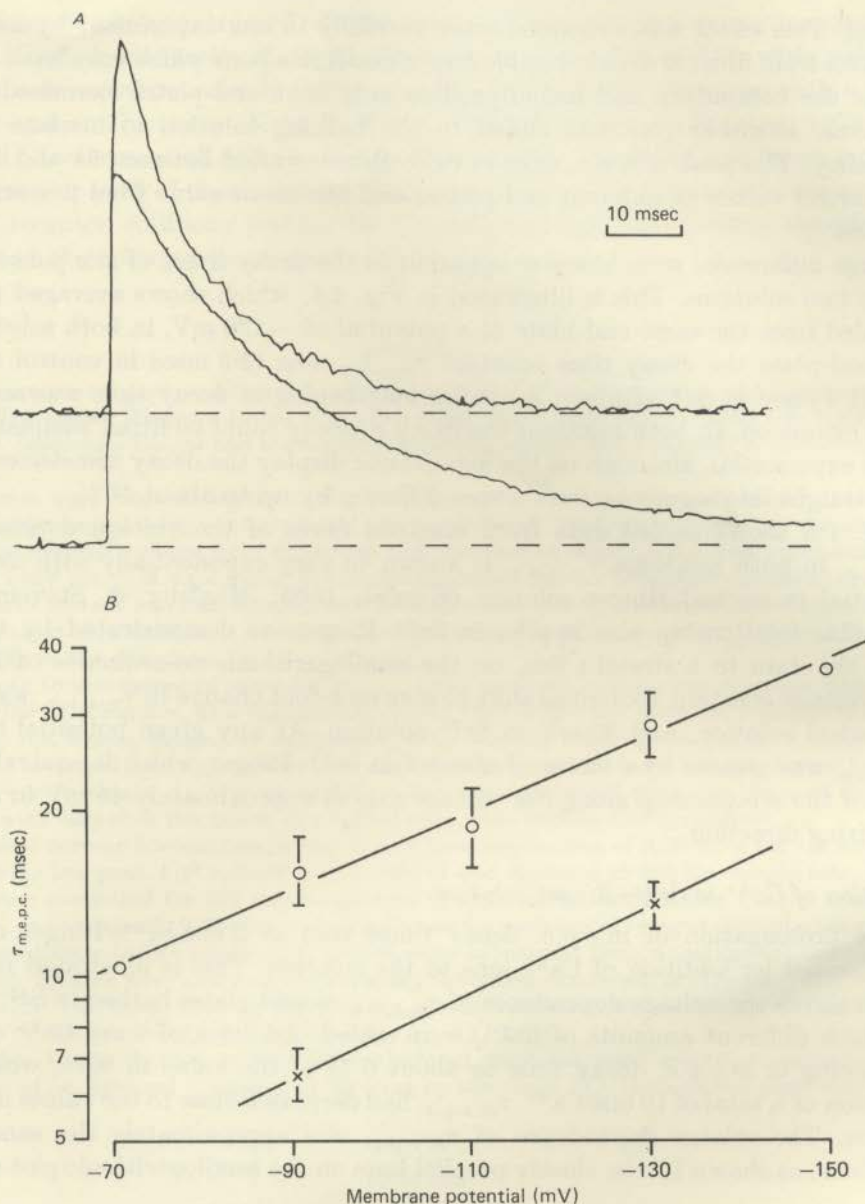
Fig. 1. *A*, tracings of computer averaged m.e.p.c.s, recorded at a clamp potential of −130 mV. The upper trace is from an end-plate bathed in control Ringer solution, and the lower trace in 2 mM-Sr^{2+}-Ringer. Both records have been scaled to the same peak height, and are displaced for clarity. Control record is an average of seven m.e.p.c.s with a mean peak size of 1·62 nA, and the Sr^{2+} record is an average of fifteen m.e.p.c.s with a mean amplitude of 1·78 nA. Records are from different fibres in the same muscle. Temperature 6–6·5 °C for both records. *B*, membrane potential dependence of m.e.p.c. decay time constant $\tau_{m.e.p.c.}$, measured in control ($\times$) and 2 mM-Sr^{2+}-Ringer ($\bigcirc$) solutions. Error bars give ±1 S.D. and the results were obtained from a total of nineteen fibres from three muscles. Points without error bars are mean values of between one and three runs. Lines were fitted by eye.
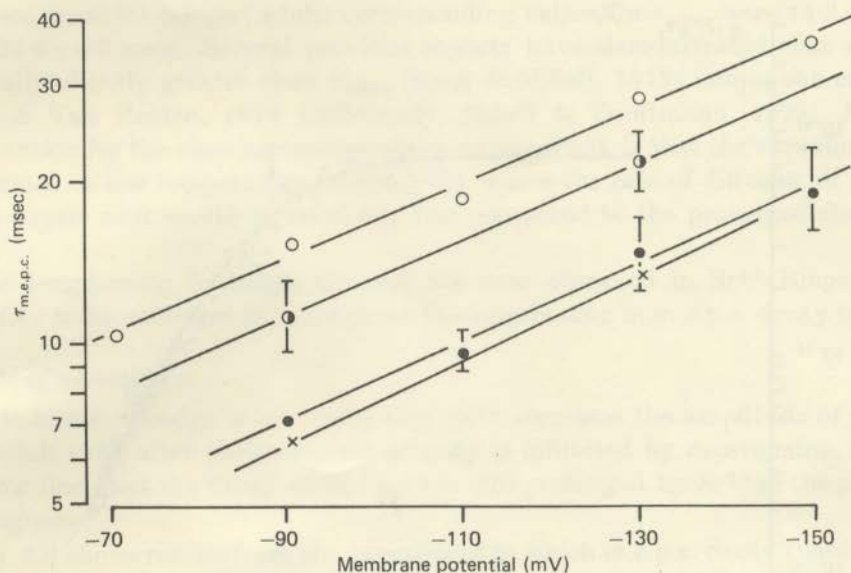
Fig. 2. Membrane potential dependence of m.e.p.c. decay time constant $\tau_{m.e.p.c.}$ showing the effect of adding Ca^{2+} ions to the 2 mM Sr^{2+}–Ringer solution. Values in control Ringer ($\times$) and 2 mM-Sr^{2+}–Ringer ($\bigcirc$) are the same as in Fig. 1*B*, and error bars for these points have been omitted for clarity. Points marked (◑) and (●) are values obtained after adding respectively 1 and 10 mM-$CaCl_2$ to the Sr^{2+}–Ringer solution. These points were obtained from a total of seven fibres from one muscle. Control and 2 mM-Sr^{2+} data were obtained from the same, and two additional muscles. Error bars are ± 1 s.d.

Lorentzian component, $S(f) = S(O)/(1 + (f/f_c)^2)$; where $S(f)$ and $S(O)$ are the spectral densities at frequencies f and O Hz respectively, and f_c is the cut-off frequency at which the spectrum has decreased to half the zero frequency asymptote.

Estimates of the single channel conductance, γ, were calculated from the total spectrum variance after correcting for the low pass filtering (Colquhoun, Large & Rang, 1977), taking a value of 0 mV for the equilibrium potential in both solutions. There was little difference in γ between control and Sr^{2+} solutions. Mean values from two muscles were (control) 18.39 ± 3.3 pS (twelve), and (2 mM-Sr^{2+}) 19.7 ± 3.9 pS (fifteen).

The mean channel lifetime, τ_{noise}, was obtained from the cut-off frequency of the spectra, as described in the methods. Fig. 3*B* shows the voltage dependence of τ_{noise}, plotted on semi-logarithmic co-ordinates, for end-plates bathed in control and 2 mM-Sr^{2+}–Ringer. The voltage sensitivity of the channel life-time was similar in the two solutions; voltage constants were 56 mV in control, and 63 mV in Sr^{2+} Ringer. At any given voltage, τ_{noise} in Sr^{2+}–Ringer was greater than in control solution by a factor of approximately 1.9.

The estimates of the mean channel life-time obtained by noise analysis are in good agreement with those obtained from m.e.p.c. decay time constants. For example, in six fibres where estimates of τ were made by both techniques at the same end-plates, mean value of $\tau_{m.e.p.c.}$ at -130 mV were 14.8 ± 1.9 msec in control, and $25.7 \pm$
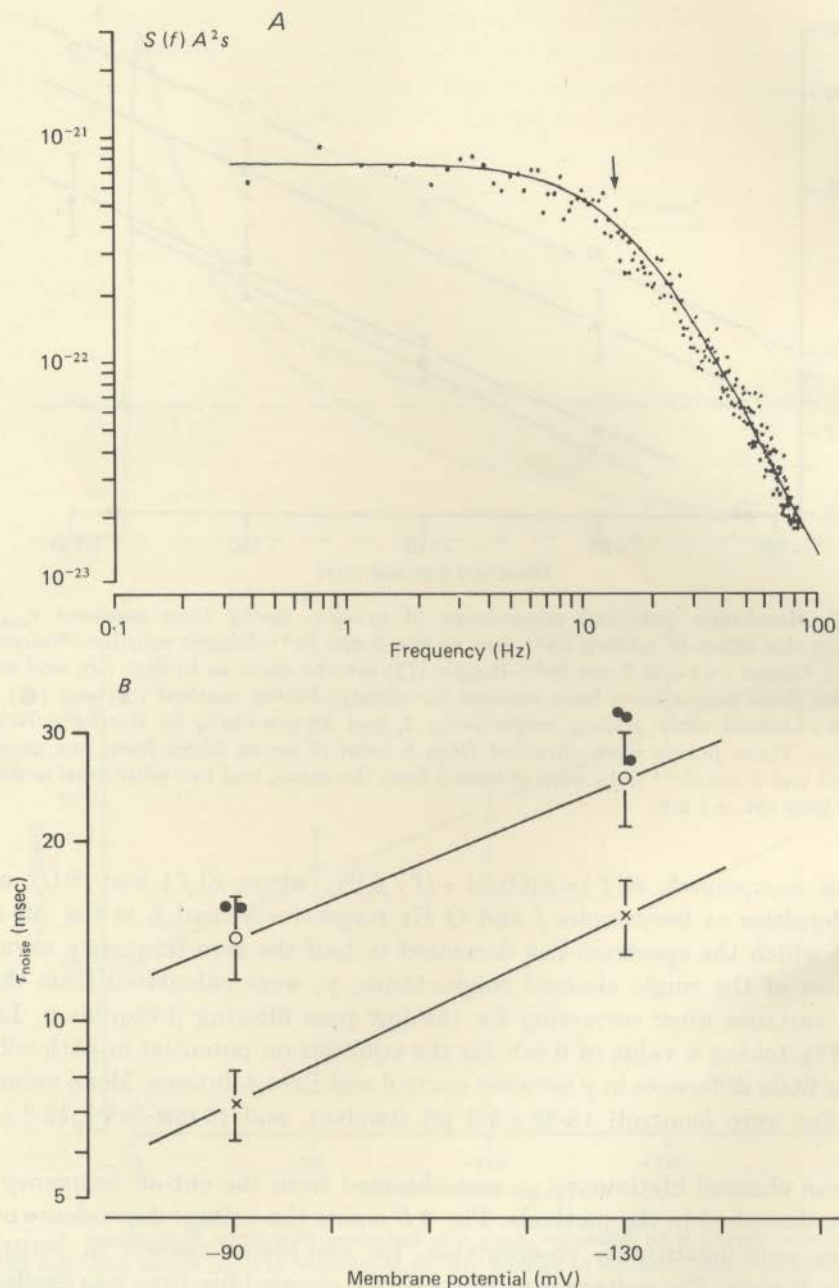
Fig. 3. A, energy spectrum of ACh current noise, obtained from an end-plate bathed in 2 mm-Sr^{2+}–Ringer. The spectrum is an average of nine runs at one end-plate (total of approximately 100 data blocks of 512 samples). Control noise spectra taken immediately before ACh application were subtracted point by point to remove background contributions from non-receptor membrane currents and electronics. The half-power frequency for the curve fitted to the data is marked by the arrow, and is 10·4 Hz. Clamp potential was −90 mV. Mean ACh induced end-plate current during the runs was 26·8 nA. B, voltage dependence of the single channel lifetime τ_{noise}, derived from noise spectra. Points shown were obtained in control solution (×), 2 mm-Sr^{2+}–Ringer (○), and 2 mm-Sr^{2+} + 3 µm neostigmine (●). Points with error bars (± 1 s.d.) are mean values from six to eight end-plates from two muscles. Data in neostigmine Ringer are individual measurements from one muscle.

3·7 msec (in Sr^{2+})–Ringer, whilst corresponding values for τ_{noise} were $14\cdot3 \pm 2\cdot3$ msec and $25\cdot6 \pm 4\cdot2$ msec. Several previous reports have demonstrated that $\tau_{m.e.p.c.}$ is generally slightly greater than τ_{noise} (Katz & Miledi, 1973; Colquhoun *et al.* 1977; Gage & Van Helden, 1979; Cull-Candy, Miledi & Trautmann, 1979). A possible explanation for the close agreement which we observed, is that the experiments were performed at low temperature (about 5 °C), where the rate of diffusion of ACh from the synaptic cleft would be relatively fast compared to the prolonged channel life-time.

The lengthening in single channel life-time observed in Sr^{2+}–Ringer appears therefore to be sufficient to account for the lengthening in m.e.p.c. decay time.

Effects of neostigmine

It is known (Dodge *et al.* 1969) that Sr^{2+} increases the amplitude of miniature potentials even after cholinesterase activity is inhibited by neostigmine. Similarly, we now find that the decay of m.e.p.c.s is still prolonged by Sr^{2+} in the presence of neostigmine.

Fig. 4A shows results from one experiment in which m.e.p.c. decay times were first recorded with the muscle bathed in normal Ringer solution, and then after addition of neostigmine to a final concentration of 3 μM. This concentration would have been sufficiently high to inhibit most of the cholinesterase activity (Katz & Miledi, 1973), but in the present experiments produced only a small increase in $\tau_{m.e.p.c.}$ at -90 mV, and almost no change at -130 mV. The low temperature (5 °C) used, probably accounts for the comparatively small effect of the neostigmine. The temperature dependence of both the channel life-time, and cholinesterase activity, is probably greater than for diffusion rates, and at 5 °C loss of ACh from the synaptic cleft by diffusion probably occurred in a shorter time than the mean channel life-time. In control experiment at 21 °C a frog from the same batch showed a prolongation of m.e.p.c. decay time by a factor of about 2·5 after addition of 3 μM neostigmine.

In neostigmine treated muscles, a marked prolongation of m.e.p.c. decay time was still observed when changing from control to Sr^{2+}–Ringer (both solutions containing 3 μM neostigmine). The shapes of the m.e.p.c.s were however frequently altered in the Sr^{2+}–Ringer, and the decay phase of many m.e.p.c.s no longer resembled a simple exponential. Fig. 4B shows typical examples of m.e.p.c.s recorded from an end-plate where a high proportion of m.e.p.c.s displayed a prolonged, nearly flat, plateau, before beginning to decline. Considerable variation was encountered in the proportion of m.e.p.c.s recorded at any one end-plate which exhibited a plateau. The end-plate of Fig. 4B showed mostly plateau type m.e.p.c.s, although a few responses were of an apparently normal, approximately exponential, form (e.g. lowest trace). Some other end-plates had a much smaller proportion of plateau m.e.p.c.s, whilst in two out of a total of five muscles examined, no clear plateau type m.e.p.c.s could be observed at any end-plates examined.

The shape of the plateau type m.e.p.c.s is similar to that recorded previously, using extracellular recording, in a solution containing Sr^{2+} and neostigmine (Dodge *et al.* 1969). The m.e.p.c.s also resemble those obtained in neostigmine treated muscles, bathed in normal Ringer, in which the end-plate was compressed by pressure from an extracellular micro-pipette (Katz & Miledi, 1973). This 'pressure
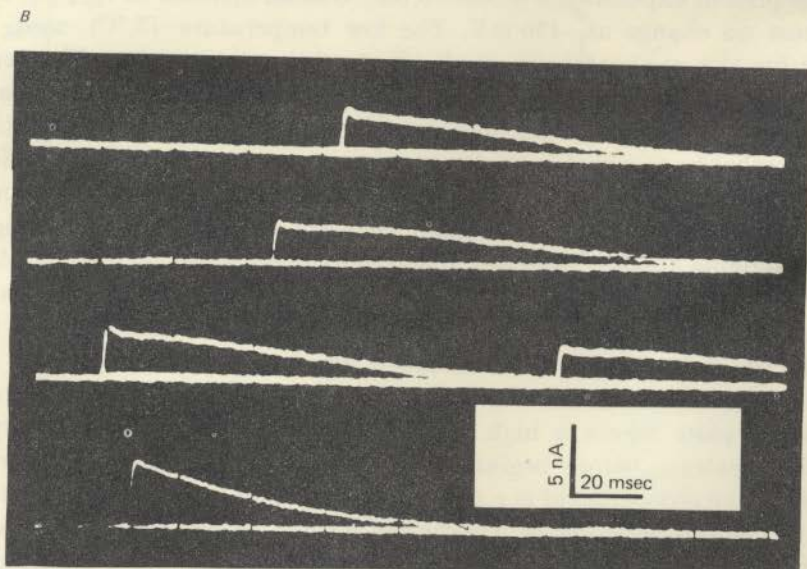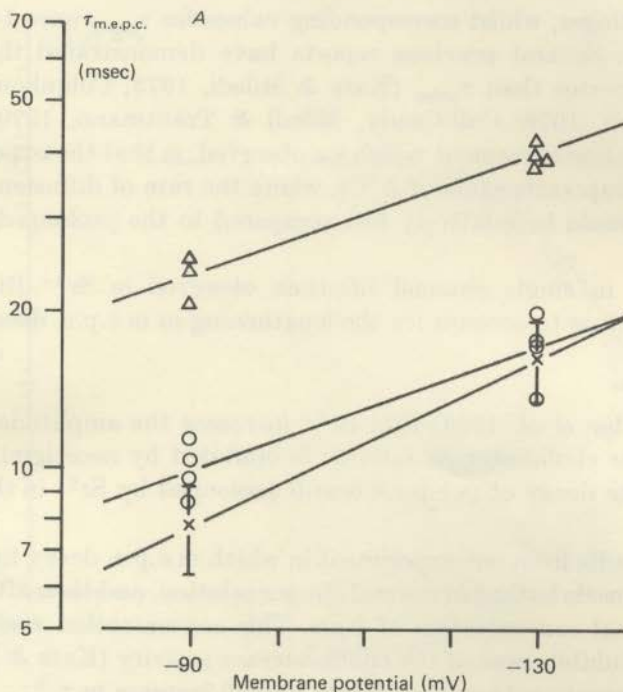
Fig. 4. A, voltage dependence of m.e.p.c. decay time $\tau_{m.e.p.c.}$, measured from end-plates bathed in control solution ($\times$), control + neostigmine ($\bigcirc$), and 2 mM-Sr^{2+}–Ringer + neostigmine ($\triangle$). Neostigmine concentration was 3 μM in both cases. Error bars are ± 1 s.D. Results are from a total of eight end-plates from one muscle. In the case of the Sr^{2+} + neostigmine data, $\tau_{m.e.p.c.}$ was measured from the exponential 'tail' of the m.e.p.c.s, beginning at about 50 % of the peak height. B, individual m.e.p.c.s recorded from an end-plate bathed in 2 mM-Sr^{2+}–Ringer containing 3 μM neostigmine. Clamp potential was −110 mV. The majority of m.e.p.c.s recorded at this end-plate showed plateaus, although a few were of an apparently normal form (e.g. lowest trace). Another characteristic feature of m.e.p.c.s in this solution, which can be seen on some traces, was an initial sharp decline following the rising phase, and preceding the plateau.

artifact' was presumably not the cause of the plateau type m.e.p.c.s in our experiments, since they could be recorded from fibres at which the end-plate was visually identified on the opposite side of the fibre to that in which the microelectrodes were inserted.

The falling phase of m.e.p.c.s subsequent to a plateau top could be fitted well by a single exponential. The upper line in Fig. 4*A* shows data from end-plates in $Sr^{2+}+$ neostigmine Ringer, in which $\tau_{m.e.p.c.}$ was estimated from only the tail of the responses, beginning at about 50 % of the peak height. The voltage dependence of $\tau_{m.e.p.c.}$ was similar in Sr^{2+} and control Ringer solutions containing neostigmine (voltage constants; control, 62 mV; Sr^{2+}, 71 mV), and the value of $\tau_{m.e.p.c.}$ at any given potential was longer in Sr^{2+}–Ringer by a factor of about 2·4.

Estimates of the mean channel life-time from noise analysis in Sr^{2+}–Ringer containing neostigmine are included in Fig. 3*B*. The values shown are slightly higher than the mean values in Sr^{2+}–Ringer without neostigmine, but the difference may not be significant, since the data were obtained from a single muscle, which showed a larger than average value of $\tau_{m.e.p.c.}$ in both control and Sr^{2+}–Ringer.

DISCUSSION

Our results show that approximately equimolar substitution of Sr^{2+} ions for Ca^{2+} (2 mM-Sr^{2+} for 1·8 mM-Ca^{2+}) in the bathing solution results in a slowing of m.e.p.c. decay time by a factor of about two, and causes a very small (4–6 %) increase of m.e.p.c. peak size. The increase in miniature potential size previously observed (Dodge *et al.* 1969) may therefore be attributed largely to the prolongation of m.e.p.c. decay time.

The increase of m.e.p.c. size was parallelled by an increase (7 %) in single channel conductance derived from noise analysis, but both changes are too small to analyse with confidence. However, since both changes are small, and of an approximately equal magnitude, it is apparent that the number of end-plate channels opened by a quantum of transmitter is essentially the same in control and 2 mM-Sr^{2+} solutions. In control experiments we obtained values of $25·8 \pm 2·7$ nS for the peak conductance during an m.e.p.c., and $18·39 \pm 3·3$ pS for the single channel conductance, suggesting that the m.e.p.c. results from the opening of about 1400 ionic channels.

The slowing of m.e.p.c. decay time can be accounted for by the increase in single channel life-time, and estimates of channel life-time by noise analysis showed an increase by a factor of about 1·9 in Sr^{2+}–Ringer. The voltage dependence of both m.e.p.c. decay time, and single channel life-time were not appreciably changed in Sr^{2+}–Ringer. Moreover, the increase in m.e.p.c. decay time did not arise from any inhibition of cholinesterase activity by Sr^{2+} ions since, (*a*) the increase in single channel life-time was sufficient to account for the increase in $\tau_{m.e.p.c.}$, (*b*) radiochemical assays of cholinesterase activity of frog sartorius muscles showed no significant difference when carried out in solutions containing 1·8 mM-Ca^{2+} or 2 mM-Sr^{2+}, and (*c*) a similar increase in $\tau_{m.e.p.c.}$ was seen with Sr^{2+} substitution when cholinesterase activity was initially blocked by neostigmine. These experiments were performed at low temperature (5 °C), where cholinesterase activity would in any case be reduced.

The reason for the increase in single channel lifetime in Sr^{2+}–Ringer is not clear. One effect of the change in divalent cation species would be to alter the surface charge density at the end-plate membrane (Gilbert, 1971; Ohmori & Yoshii, 1977). thus altering the potential gradient across the membrane and so affecting the channel life-time. The expected shift in potential from this source is however much smaller than the shift of 35–45 mV required to fit our observations. For example, a modified form (Hille, Woodhull & Shapiro, 1975) of the Gouy-Chapman theory of surface potential (Grahame, 1947) predicts a shift of about 4 mV in a less negative direction by substitution of 2 mM-Sr^{2+} for 1·8 mM-Ca^{2+}. Experimental observations of shifts in activation potential of a variety of ionic channels are in good agreement with this prediction (Hille *et al.* 1975; Ohmori & Yoshii, 1977). The observed shortening of $\tau_{m.e.p.c.}$ resulting from addition of Ca^{2+} ions to 2 mM-Sr^{2+}–Ringer is also in contrast to the behaviour expected from surface charge effects. Addition of 10 mM-Ca^{2+} would be expected to cause the surface potential to become about 15 mV less negative (Hille *et al.* 1975), thus leading to an increase in $\tau_{m.e.p.c.}$. The decrease in $\tau_{m.e.p.c.}$ with increased Ca^{2+} concentration is however similar to results we have obtained by increasing the Ca^{2+} concentration in normal Ringer solution (Bregestovski, Miledi & Parker, 1979), although an increase in $\tau_{m.e.p.c.}$ has also been reported under these conditions (Cohen & Van der Kloot, 1978).

Although changes in membrane surface charge may well influence the end-plate channel life-time, our results indicate that the species and concentration of divalent cations in the bathing medium must have some additional, and quantitatively larger, effect upon the channel life-time. The nature of this effect is at present unclear. Possible mechanisms by which the life-time might be altered include, (i) an effect of Sr^{2+} ions on the lipid environment of the channels, (ii) changes in channel properties resulting from Sr^{2+} ions permeating the channels (cf Van Helden, Hammill & Gage, 1977; Gage & Van Helden, 1979), (iii) an action of external Sr^{2+} ions on channel properties, or (iv) changes in the rates with which ACh interacts with the receptor.

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