Br. J. Pharmac. (1973), **47**, 363–376.

Influence of chloride ions on changes in membrane potential during prolonged application of carbachol to frog skeletal muscle

D. H. JENKINSON AND D. A. TERRAR*

Department of Pharmacology, University College, London WC1E 6BT

Summary

1. Micro-electrodes were used to follow changes in the membrane potential at the end-plate region of single fibres in narrow strips of frog skeletal muscle exposed to carbachol applied in continuously flowing Ringer solution containing tetrodotoxin (200 nM) and neostigmine (3 μM).

2. The depolarizations elicited by carbachol (5–20 μM) usually developed in two phases, the first of which was generally complete within 30 s whereas several min were required for the second.

3. Repolarization after carbachol also occurred in two phases, the second of which outlasted the time needed to clear the bath, and varied with the magnitude and duration of the depolarization which carbachol had caused.

4. These findings could best be explained in terms of the consequences of net entry of chloride ions into the fibre during the depolarization caused by carbachol. This hypothesis is supported by three lines of evidence:

(a) Replacement of the chloride content of the Ringer solution by the less permeant anion isethionate abolished the slow phases of the carbachol response.

(b) Reduction of chloride permeability (by lowering pH) caused rapid repolarization during the recovery period after carbachol.

(c) When the membrane potential was clamped at the resting level throughout the action of carbachol, so avoiding chloride redistribution, the clamping current records did not show the slow phases attributed to chloride movement.

5. Chloride redistribution contributes to the gradual spread of depolarization during prolonged applications of depolarizing agents to skeletal muscle. It also complicates the interpretation of the dose-response relationship, and may make it more difficult to assess the extent to which the receptors become desensitized during the action of agonists applied in the bath.

Introduction

Quantitative studies of dose-response relationships are often made *in vitro*, and necessitate exposing the tissue in question to different concentrations of agonist for long enough for a steady effect to be attained. The time needed for this varies greatly from preparation to preparation, ranging from a few seconds, as with thin strips of intestinal muscle (cf. Cuthbert & Dunant, 1970), to several minutes, as

* Present address: Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33152, USA.

when an isolated electroplaque from *Electrophorus* depolarizes in response to acetylcholine or carbachol (Higman, Podleski & Bartels, 1963; Karlin, 1967a; Changeux & Podleski, 1968).

A possible complication with tissues which respond relatively slowly is that the effectiveness of agonists (in particular those which act by modifying membrane permeability) may alter during the course of the application as a consequence of changes in the concentrations of inorganic ions within the cells, so that the final response may provide a misleading indication of receptor activation. Previous discussions of this possibility have been concerned mainly with the cation movements which occur during the action of carbachol on the electroplaque (Karlin, 1967a, although see Blumenthal & Changeux, 1970). However, anion movements have also to be considered, particularly with tissues (e.g. both smooth and skeletal muscle) which have an appreciable permeability to chloride ions. The present experiments were done to assess the importance of this factor in skeletal muscle, and show that it can indeed have a marked influence on the response to maintained concentrations of depolarizing agents such as carbachol. Certain consequences of this finding, in particular for the interpretation of the dose-response relationship, and for the assessment of desensitization, have also been examined.

Methods

The experiments were done throughout the year at room temperature (20–23° C) with skeletal muscle from *Rana temporaria*. A narrow strip (1–2 mm in width) was dissected from the edge of the sartorius and mounted under slight tension over a curved perspex support in the bottom of a channel (width 2 mm, depth 4 mm) milled from a perspex block. In a few experiments whole sartorius muscles were used, held in a channel 10 mm wide and 4 mm deep. In either case the preparation was mounted with the deep surface of the muscle uppermost, and with the pelvic tendon closest to the inflow.

Bathing fluids

A continuous stream of Ringer solution flowed through the channel from a reservoir 60 cm above the muscle bath. The flow rate was 14 ml min^{-1}, corresponding to a linear flow of 3·5 cms^{-1} at the shallowest part of the channel (where the perspex support for the muscle reduced the depth to 2 mm). A multiple tap connected to the bath by narrow silicone rubber tubing allowed the composition of the bathing fluid to be changed rapidly, the delay from turning the tap to arrival of a new solution over the recording site in the channel being less than 5 seconds.

The standard Ringer solution contained (mM): NaCl, 116; KCl, 2·0; $CaCl_2$, 1·8; Na_2HPO_4, 1·92; NaH_2PO_4, 0·48. In some experiments chloride was replaced by the less permeant anions isethionate and methylsulphate to give a solution with the composition (mM): Na isethionate, 116; K methylsulphate, 2·0; $CaSO_4$, 2·4; Na_2HPO_4, 1·92; NaH_2PO_4, 0·48. When the pH (normally 7·2–7·3) was to be altered, the phosphate buffer was replaced by one containing *N*-acetylglycine (2·0 mM) and tris maleate (2·0 mM), and the pH brought to the required value with NaOH (see Hutter & Warner, 1967a).

In most experiments tetrodotoxin (100 nM) was added to all solutions, to prevent twitching when carbachol was applied. Neostigmine (3 μM) was also included in

order to increase the size of the miniature end-plate potentials, and so facilitate localization of the end-plate regions of the fibres. Similar results were obtained in control experiments in which either drug was omitted.

Electrical recording

Membrane potentials were recorded in the conventional way with micro-electrodes filled with either potassium chloride (3 M) or potassium citrate (2 M, acidified with citric acid to pH 6); resistances ranged from 15–30 MΩ. A chlorided silver wire mounted in Ringer-agar served as a stable bath electrode, although it was necessary to allow for a change of 8 mV in junction potential on replacing the chloride content of the bathing fluid by isethionate. Potential differences between the micro-electrode and the bath electrode were displayed, via a differential cathode follower input, on an oscilloscope (Tektronix 502A) with a pen recorder (Devices M2) connected in parallel. The records shown were obtained with the latter which had an adequate frequency response (DC to 70 Hz) for the present purposes, although the miniature end-plate potentials and to a lesser extent the responses to transient acetylcholine applications (Fig. 6), will have been attenuated.

In some experiments, a second micro-electrode was used to pass current to 'voltage-clamp' the membrane during applications of carbachol in the bathing fluid. This electrode was filled with potassium methylsulphate (2 M, acidified to pH 6) and was arranged in a feed-back circuit of the kind described by Kordaš (1968). One of the vertical amplifiers of the oscilloscope provided the clamping current which was monitored by an operational amplifier connected as a current-to-voltage transducer.

In other experiments acetylcholine was applied to sensitive spots at the end-plate region by iontophoresis from a micro-pipette containing acetylcholine (2 M). as described by Nastuk (1953) and by del Castillo & Katz (1955).

Materials

Solutions were made up in glass-distilled water with salts of Analar grade, except for sodium isethionate and potassium methylsulphate which were laboratory grade reagents obtained from Koch-Light and B.D.H. respectively. Carbachol (carbamoyl choline chloride, B.D.H.) was made up as a stock solution (10^{-2} M) just before each experiment. Other substances used included neostigmine methylsulphate (Roche), tris maleate and *N*-acetylglycine (both from Sigma) and tetrodotoxin (Sankyo).

Results

Figure 1 illustrates the effect of a 3 min application of carbachol (10 μM) on the membrane potential at the end-plate region of a skeletal muscle fibre. Two particular features of the record deserve comment. First, the response was still increasing at the end of the 3 min period, even though the bathing fluid had been changed at a rate such that the full concentration of carbachol should have been attained within 15 seconds. Second, the recovery of the membrane potential on washing out carbachol occurred in two phases, the first of which was over within 20 s, the time required to clear the bath, whereas the second required more than 10 min for completion.

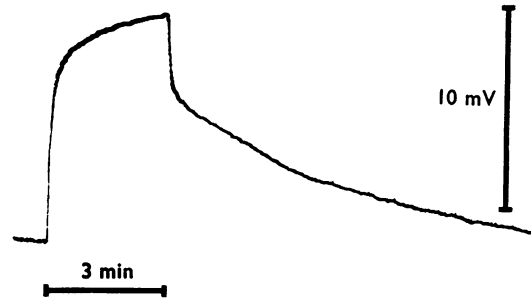

FIG. 1. Pen recorder trace showing the effect of carbachol (10 μM, applied for 3 min) on the membrane potential at the end-plate region of a frog skeletal muscle fibre (resting potential −89 mV). The preparation (a thin strip cut from the edge of the sartorius) was bathed at 23° C in a continuously flowing solution containing neostigmine (3 μM) and tetrodotoxin (100 nM) throughout. From experiment of Fig. 5.

At first it was thought that the slow decline of the response might reflect the outward diffusion of carbachol 'trapped' in the interspaces between fibres deeper within the muscle. Another possibility considered was that a fraction of the receptors remained active after carbachol had been washed out, although this seemed unlikely in the light of experiments of del Castillo & Katz (1957, see also Katz & Miledi, 1965) in which the iontophoretic method of drug application was used to demonstrate that the effects of acetylcholine and carbachol are very rapid in both onset and decline.

A more satisfactory explanation was found by considering the change in the chloride content of the fibre during the action of carbachol. This will now be described in some detail, both to indicate how the observed effects could be accounted for, and to introduce the main series of experiments made to test the hypothesis.

Although acetylcholine is known to depolarize the end-plate region of skeletal muscle by increasing the permeability of the membrane to cations rather than anions (Fatt & Katz, 1951 ; Takeuchi & Takeuchi, 1960), a substantial net influx of chloride ions can nevertheless be expected if the depolarization is maintained. It is well established that skeletal muscle fibres normally contain little chloride, the intracellular concentration, $[Cl]_i$, being set by the potential difference across the membrane in such a way that the chloride equilibrium potential (E_{Cl}) is close to the resting potential (see Hodgkin, 1958 ; Hodgkin & Horowicz, 1959). However, when a depolarizing agent such as acetylcholine or carbachol is applied, the resulting increase in cation permeability causes the membrane potential to fall below E_{Cl}. The result will be that the inward sodium current (I_{Na}) will be opposed not only by an outward potassium current (I_K) but also by an outward chloride content (I_{Cl}) because of net influx of chloride ions. In the absence of such a chloride current the depolarization produced by the agent would be greater (see later). However, as the influx of chloride continues the internal chloride concentration will gradually increase, causing E_{Cl} to become less negative so that the driving force for chloride influx will diminsh. A greater fraction of inward I_{Na} will now have to be balanced by outward I_K with the result that the depolarization will gradually increase, as indeed was observed. This occurs even though the ionic permeabilities may remain unchanged.

After withdrawal of the drug, the permeability to sodium and potassium will decline toward their values in the resting tissue. However, since $[Cl]_i$ is still raised,

E_{Cl} is now *less* negative than the value determined by P_K and P_{Na}. The membrane potential therefore assumes an intermediate value, full repolarization occurring only when $[Cl]_i$ has returned to its original low level. An additional factor which will slow repolarization is the decline in potassium permeability which occurs when the electrochemical gradient for potassium is directed outwards (Katz, 1949; Hodgkin & Horowicz, 1959; Adrian & Freygang, 1962), as it will be at the end-plate and in surrounding parts of the fibre during recovery from the action of the depolarizing agent.

This hypothesis was examined in four different kinds of experiment.

1. Complete replacement of the chloride in Ringer solution by impermeant anions such as isethionate and methylsulphate would be expected to abolish the slow phases in the onset and decline of the response to carbachol. This was indeed observed, as illustrated in Fig. 2 (see also Fig. 3 *d*, *e*; Figs. 4–7).

2. Extending the duration of the carbachol application should cause the slow phase of subsequent repolarization to become more marked, since higher values of $[Cl]_i$ should be attained. This was also found (Fig. 3).

3. A further prediction of the hypothesis is that any procedure which reduces P_{Cl} relative to P_{Na} and P_K should cause the membrane to repolarize if tested during the slow recovery of potential after a long application of carbachol. The work of Hutter & Warner (1967a, b) has shown that such a differential change in the

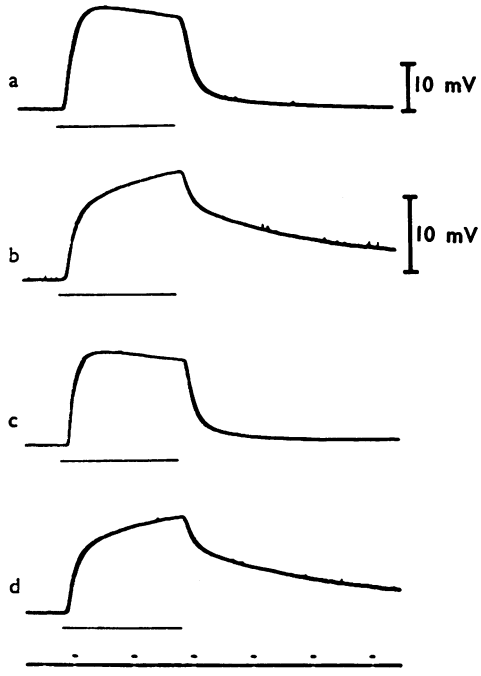

FIG. 2. Effect of replacing the chloride content of Ringer fluid by isethionate on the response to carbachol (20 μM, during bar). Record *a* was taken in isethionate solution, after which normal Ringer fluid was restored and carbachol retested, *b* after an equilibrium period of 32 minutes. Chloride was again replaced by isethionate and carbachol applied *c*. Finally normal Ringer solution was reintroduced, and the response to carbachol is shown in *d*. Note the marked influence of chloride on the time-course of depolarization, and the reversibility of the effect. The voltage calibration for *a* also applies to *c* and that for *b* to *d*. Time marker, min. Resting potential in chloride solution, −91 mV; in chloride-free, −93 mV.

membrane permeability of skeletal muscle can be achieved by lowering the pH of the bathing fluid to about 5·0–5·3. The experiments illustrated in Fig. 4a and b showed that reduction in pH indeed caused repolarization. In keeping both with the conclusions of Hutter & Warner and with the present hypothesis, there was little if any effect on the membrane potential of muscles equilibrated in chloride-free solution (Fig. 4c).

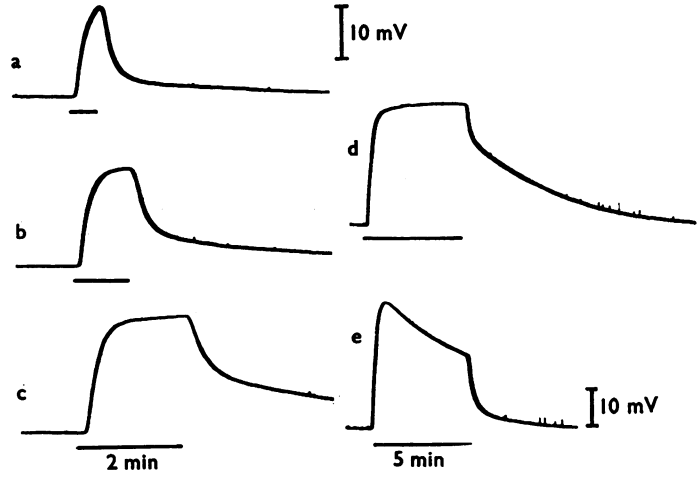

FIG. 3. Effects of varying the duration of application of carbachol (20 μM) on the time course of repolarization after the drug. Records *a–d* in Ringer fluid, *e* in isethionate. The voltage calibration for *a* also applies to *b–d*. Paper speed reduced for *d* and *e*. Note the progressive increase in the slow phase of repolarization from *a* to *d*, and its absence in *e*. Membrane potential, −98 mV in Ringer solution, −100 mV in isethionate.

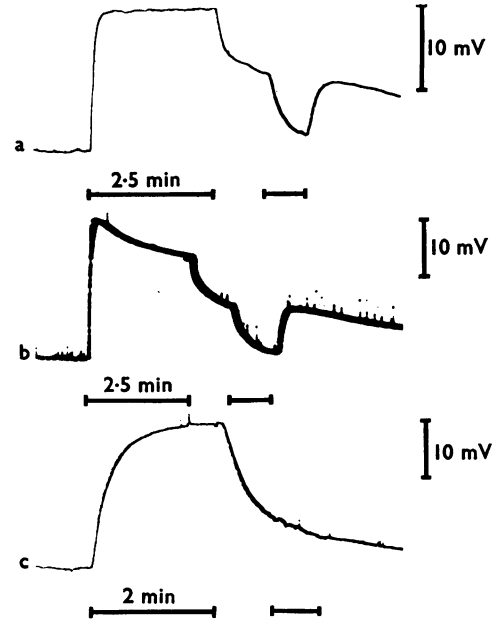

FIG. 4. Effect of lowering the pH of the bathing fluid from 7·2 to 5·5 (for the period shown by the second bar under each record) on membrane potential during recovery from carbachol (applied during the first bar at 20 (*a*, *c*) or 40 (*b*) μM). Reduction in pH causes repolarization in normal Ringer fluid (*a*, *b*) but not when chloride has been replaced by isethionate (*c*). Separate experiments.

4. In a final test, a second micro-electrode inserted in the end-plate region close to the recording electrode was used to pass the current required to 'clamp' the membrane potential at the resting level throughout the application of carbachol. Under this condition there should, of course, be no redistribution of chloride, and the clamping current should provide a more accurate measure of the effect of carbachol on membrane permeability. Thus the current record should not show the characteristic slow phases evident in the potential records. A further prediction is that differences between the time courses of (a) the clamping current and (b) the unclamped membrane potential, should become less marked in the absence of external chloride. It is, of course, well known that a completely effective clamp is difficult to achieve, mainly because current passage from a single point source such as a micro-electrode can provide only an approximation to the ideal of a uniform voltage clamp over the whole end-plate region (see discussions by Takeuchi & Takeuchi, 1959 ; Kordaš, 1968 and Auerbach & Betz, 1971). Another complication is that technical factors, e.g., capacitative interactions between the electrodes, impose a limit on the gain which can be used in the feed-back circuit, with the consequence that in the present experiments the membrane potential still fell during the action of carbachol, although by less than 10% of the value recorded in the unclamped condition. This degree of clamping was considered adequate for the purpose intended, and Fig. 5 illustrates the results obtained in such an experiment. It may be seen that the time course of the clamping current indeed differed in the expected way from the potential record from the unclamped fibre, and further that the difference between the two types of record became much less marked when the muscle had been equilibrated in chloride-free solution. An incidental observation

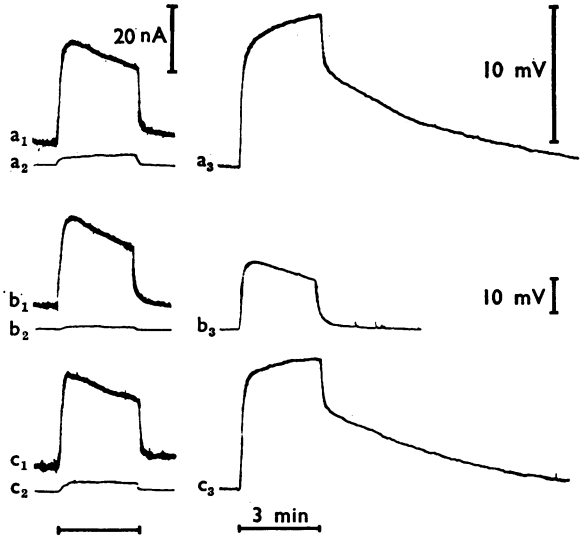

FIG. 5. Comparison of two measures of the action of carbachol on skeletal muscle. a_1, b_1 and c_1 show the currents necessary to ' clamp ' the membrane potential at the end-plate region during the presence of carbachol (10 μM) applied first in Ringer fluid (a_1) then in chloride-free solution (b_1) and finally (c_1) with the muscle in normal Ringer again. a_3, b_3 and c_3 illustrate the corresponding changes in membrane potential in the unclamped fibre. a_2, b_2 and c_2 show the residual potential changes occurring during the clamp owing to technical limitations in the procedure (see text). The 10 mV calibration (upper right) applies to a_2, a_3, c_2 and c_3. Gain reduced for b_2 and b_3. The current calibration (upper left) applies to a_1, b_1 and c_1. Same fibre (resting potential -89 mV in both normal and chloride-free solution) with electrodes *in situ* throughout.

was that there was no consistent change in clamping current on replacing chloride by isethionate, showing that the effect of carbachol on cation permeability is not markedly anion dependent. Similar results were obtained in four similar though less complete experiments.

Some consequences of chloride redistribution during the action of carbachol

Apparent time-course of desensitization

There is evidence to suggest that prolonged applications of agonists can cause receptors to become 'desensitized', i.e. to lose their responsiveness in some as yet little understood fashion. This is readily observed with isolated frog skeletal muscle in which the onset of desensitization is evident as a gradual decline in the depolarization elicited by acetylcholine or carbachol (Fatt, 1950; Thesleff, 1955a; Katz & Thesleff, 1957; Magazanik & Vyskočil, 1970; Nastuk & Parsons, 1970; Rang & Ritter, 1970). The present findings suggest that unless this 'spontaneous' repolarization is very fast (as it can be when drugs are applied iontophoretically, see Katz & Thesleff, 1957), the rate at which the membrane potential recovers in the continued presence of the drug may provide a rather poor measure of the extent of desensitization. This is because the concurrent redistribution of chloride ions will tend to influence the membrane potential in the opposite direction (cf. discus-

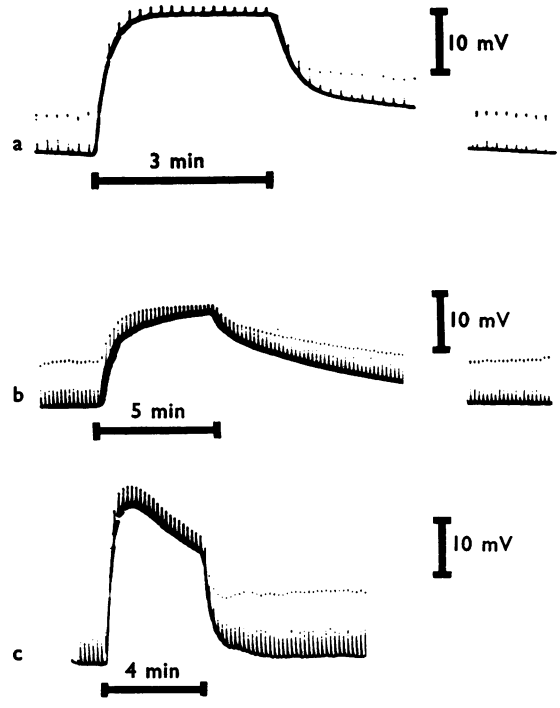

FIG. 6. Changes in the response to iontophoretically applied acetylcholine during the action of bath-applied carbachol (20 μM). The transient depolarizations caused by the test pulses of acetylcholine appear as vertical deflections of the trace. Separate experiments: in those of *a* and *b* the bathing fluid contained the normal concentration of chloride ions (although in *b* all but 0·18 mM of the external calcium was replaced by magnesium in an attempt to reduce local contractures), whereas in *c* chloride was replaced by isethionate. For further details, see text.

sion in relation to Fig. 1). These opposing tendencies can be expected to balance under certain conditions, and this, it is thought, can account for the relatively stable 'plateau' of depolarization often observed on applying intermediate concentrations (20–30 μM) of carbachol (see Figs. 3d, 4a, 6a, 7a_1, b_1, c_1, d_1, 8f). The finding in our voltage clamp experiments that the clamping current began to decline at a time when the depolarization recorded from the unclamped fibre was still increasing is clearly in keeping with this suggestion.

Masking of desensitization by anion redistribution was examined more directly by using the response to brief pulses of acetylcholine from a micro-pipette to follow changes in receptor sensitivity during the presence of carbachol in the bathing solution. Figure 6 illustrates the results obtained in three such experiments. It is seen in (a) that the response to acetylcholine pulses continued to fall (presumably because of the onset of desensitization) even when the depolarization produced by bath-applied carbachol had reached a plateau. In Figure 6b the discrepancy is still more marked: the response to the test pulse fell steadily even though the carbachol depolarization was still rising at the end of the application period.

While these results provide further qualitative support for the hypothesis which has been outlined, detailed analysis of such experiments is complicated by two factors. First, part of the reduction in the voltage change produced by the test pulses of acetylcholine is due to the increase in membrane conductance caused by carbachol applied in the bath (see del Castillo & Katz, 1955). Second, the response to both carbachol and acetylcholine will be influenced by the rise in the intracellular chloride concentration. This latter complication can be avoided by using a chloride-free bathing solution, and indeed the discrepancy between the apparent extent of desensitization (as gauged on one hand by the fall in response to acetylcholine pulses and on the other by the depolarization produced by bath-applied carbachol) was then found to be much smaller, and largely accountable for in terms of the shunting of the membrane resistance in the way discussed by del Castillo & Katz. Thus, in the record of Fig. 6c it can be seen that during the last 2·5 min of the drug application the depolarization produced by carbachol fell from 28 to 20 mV, presumably as an increasing proportion of the receptors became desensitized. The response to test pulses of acetylcholine is now influenced in opposing ways: fewer non-desensitized receptors remain, but of these the fraction activated by acetylcholine will tend to produce a greater effect because the 'shunt' set up by carbachol is becoming smaller, as indicated by the fall in the carbachol depolarization. Reference to the relevant equation derived by del Castillo & Katz (1955, see discussion in relation their Fig. 15) suggests that if the latter factor alone had operated (i.e. if the shunt caused by carbachol had fallen because of some factor other than receptor desensitization), the response to the test pulse would have *increased* by about 30% as the membrane repolarized in the experiment of Figure 6c. If, however, the decline in response to carbachol is attributable to desensitization, as seems likely, it may be estimated from the fall in the carbachol depolarization that the effectiveness of the acetylcholine pulses in increasing membrane conductance would have declined by about 40% in the same period. Since these opposing influences are almost matched only a small fall in the acetylcholine potential should occur, as was indeed observed (Fig. 6c). Two other experiments were analysed in the same way, and provided similar results.

Spread of depolarization

When carbachol is first applied, the ensuing depolarization is greatest at the end-plate regions of the muscle and falls away steeply in either direction along the fibre. If the application is maintained, increasing amounts of chloride will enter the fibre not only at the end-plate but also at more distant points at a rate depending mainly on P_{Cl} and on the difference between E_{Cl} and the existing membrane potential (displaced from the resting value by current flow from the end-plate). The resulting rise in intracellular chloride along the fibre will have the effect of causing the depolarization to 'spread', so that the membrane potential some distance from the end-plate may continue to fall (as E_{Cl} at the point gradually becomes less negative) even if the potential at the end-plate has reached a steady value.

Evidence on this point was obtained by placing two micro-electrodes in a fibre, one at the end-plate and the other several mm away. Typical records taken during the action of carbachol are shown in Fig. 7, where it may be seen that the time course of the depolarization at the distant electrode differed in two main respects: (1) the potential was still falling rather rapidly at the end of the drug period, and (2) the slow phase of repolarization was relatively more marked. If, as is thought, both features are a consequence of chloride redistribution, the difference between the time courses would be expected to disappear on replacement of the chloride content of the bathing solution by an impermeant anion. This was also observed, as illustrated in Fig. 7e and f.

The dose-response relationship

Figure 8 compares the end-plate depolarizations produced by a range of concentrations of carbachol applied first in chloride-free (left) and then in normal

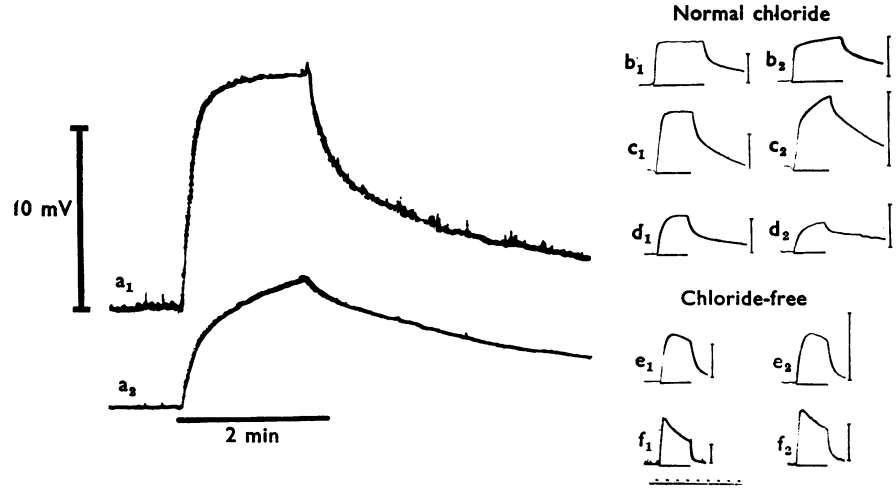

FIG. 7. *Left.* The effect of carbachol (20 μM) on membrane potential at the end-plate region (a_1) and 2·4 mm away (a_2). Simultaneous records from the same fibre (resting potential −89 mV) in a muscle bathed in normal Ringer solution. *Right.* Pairs of traces from other experiments of the same kind (note that records from the distant electrode are at higher gain). *b–d* in normal Ringer solution, *e* and *f*, chloride replaced by isethionate. Records d_1 d_2 and e_1 e_2 taken from the same fibre with the electrodes *in situ* during the change from chloride to isethionate solution. Interelectrode separation: b_1 b_2, 1·4 mm; c_1 c_2, 2·0 mm; d_1 d_2 and e_1 e_2, 2·9 mm; f_1 f_2, 3·5 mm. Voltage calibration, 10 mV for each record. Time marker, minutes.

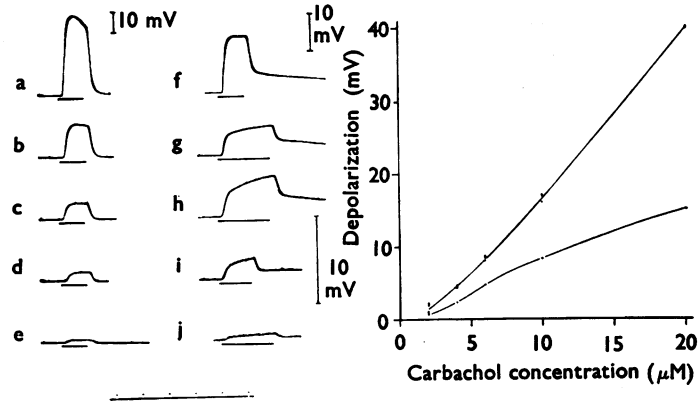

FIG. 8. *Left.* End-plate depolarization elicited by different concentrations of carbachol (*a* and *f*, 20 μM ; *b* and *g*, 10 μM ; *c* and *h*, 6 μM ; *d* and *i*, 4 μM ; *e* and *j*, 2 μM) applied first in isethionate (*a–e*) and then in normal Ringer solution (*f–j*). Voltage calibrations: that of *a* applies also to *b–e* ; that of *f* to *g*, and that of *i* to *h* and *j*. Resting potential of fibre in presence and absence of chloride, −96 and −95 mV respectively. Time marker, minutes. *Right.* The relationship between carbachol concentration and depolarization in this experiment. (Upper curve, chloride-free, lower curve, normal Ringer solution.)

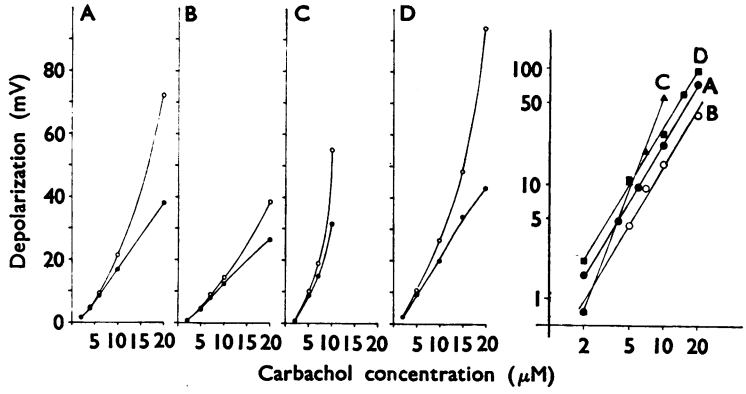

FIG. 9. A–D. Dose-depolarization relationship for carbachol applied in chloride-free solutions (lower curves). Also shown are the corresponding relative increases in membrane conductance (upper curves) estimated from the depolarizations by applying the Martin correction (see **Discussion**). Resting potentials for A (expt, of Fig. 8), B, C and D were −95, −97, −87 and −97 mV respectively. *Right.* Double logarithmic plots of conductance increase (arbitrary units) against carbachol concentration. The letters indicate the particular experiment.

Ringer solution (centre). Records *g–j* further illustrate the features discussed in relation to Fig. 1, and it is clear, particularly from *g*, *h* and *i*, that attainment of a steady depolarization would have required a still longer application, during which more chloride would have entered the fibre. It can be argued that a better measure of the action of carbachol would be given by the magnitude of the initial rapid phase of depolarization which can be distinguished in *g–i* ; this 'knee' could be expected to become more distinct were the record to be taken from an isolated fibre bathed in a very rapidly flowing solution. The response would, of course, still be anion dependent in the sense that movement of chloride into the fibre reduces the depolarization which would otherwise result from a given increase in cation permeability, as already mentioned, and the finding that the response to carbachol

was always greater in chloride-free solution is readily explained in this way. It may also be seen in Fig. 8 (right) that the dose-response relationships begin with a region of increasing slope. This was consistently observed, other examples being shown in Fig. 9.

Discussion

Our main finding is that the response of skeletal muscle to maintained application of carbachol shows certain characteristic features (in particular, slow phases in both onset and decline) which can be best explained as a consequence of changes in the chloride content of the fibres. While considerable evidence to support this suggestion has been obtained, a quantitative description has not been attempted. Among factors which would have to be taken into account are (1) the influence of fibre size (other things being equal, ion redistribution would be expected to be more rapid in smaller fibres because of the greater surface-to-volume ratio), (2) the rate at which chloride moves along the axis of the fibre as well as across the membrane, and (3) changes in the length constant, λ, of the fibres during the action of carbachol. In frog skeletal muscle the chemosensitive region may extend more than 1 mm along the fibre (see e.g. Miledi, 1960) so that depolarizing agents will reduce the resistance of a considerable area of the membrane. λ will therefore fall initially, but may recover somewhat as desensitization proceeds. This latter factor may account for the slightly less marked spontaneous decline in the depolarization recorded several mm away from the end-plate region of muscles exposed to carbachol in isethionate solution (compare records f_1 and f_2 in Fig. 7). An additional complication is that the extra-junctional receptors may differ from those close to the nerve endings not only in their pharmacological properties but possibly also in the selectivity of the ion conductance changes they produce (Feltz & Mallart, 1971a, b, but see also Peper & McMahan, 1972).

These factors have also to be borne in mind in interpreting the relationship between the concentrations of carbachol and the magnitude of the ensuing depolarization. A much discussed feature of the action of depolarizing agents on skeletal muscle (Katz & Thesleff, 1957; Jenkinson, 1960) as well as on the isolated electroplaque (Higman *et al.*, 1963; Karlin, 1967b; Changeux & Podleski, 1968) is that the dose-response curves (plotted linearly) begin with a region of increasing slope. The present results confirm the earlier observations with muscle. Depolarization is, of course, a less direct measure of receptor activation than the underlying increase in membrane conductance which can, however, be estimated in relative terms from the observed depolarization, v, by applying the factor $(1-v/V)^{-1}$, V being the resting potential minus 15 mV (Martin, 1955; see discussion by Werman, 1969). Figure 9 illustrates the outcome for the present results, the double logarithmic plot (right) suggesting that the conductance increase is proportional to the carbachol concentration raised to a power which ranged from 1·26 to 2·08, with a mean and S.E. of $1{\cdot}51 \pm 0{\cdot}04$ ($n=12$; carbachol applied at 2 to 20 μM in chloride-free solution). Such values have, of course, to be regarded with some caution in view of the complications which have already been mentioned. A further difficulty is that the applicability of the Martin correction becomes less certain for increasing depolarizations since the conductance of the non-chemosensitive part of the membrane can no longer be assumed to remain unchanged. One way of avoiding at least some of these uncertainties is to clamp the membrane potential during the action of the depolarizing

agent, and some interesting preliminary results with this technique have been obtained by Rang (1971).

Some other consequences of anion redistribution will now be considered:

(1) As already noted, changes in fibre chloride may complicate the assessment of desensitization (unless the exposure to agonist can be kept very brief, as when the iontophoretic method is used). In particular, the absence of desensitization evidently cannot be inferred from the occurrence of a stable plateau of depolarization in response to a steady concentration of agonist (cf. discussion in Ariëns, 1964) since this may merely indicate that the opposing influences of desensitization and chloride redistribution are in balance.

(2) After carbachol has been washed out, the membrane potential may recover more slowly than the membrane conductance, as first reported by Manthey (1966). Nastuk & Parsons (1970) have already suggested that the increased chloride content of the fibres may contribute to this effect, although they considered the non-linearity of the relationship between membrane conductance and potential to be the major factor involved.

(3) A further consequence is that the depolarization elicited by carbachol or acetylcholine may gradually extend from the end-plate region as fibre chloride rises. It may be recalled that Burns & Paton (1951) noted a gradual spread in the depolarization caused by the action of decamethonium on cat skeletal muscle *in vivo*, and it seems possible that chloride redistribution could be concerned in this situation as well.

There have also been reports of extra-junctional depolarization in isolated muscles exposed to acetylcholine or carbachol in the bath (Thesleff, 1955b; Ochs & Mukherjee, 1959; Portela, Perez, Vaccari, Perez & Stewart, 1970; Ras, den Hartog & Lammers, 1972). Although such findings have sometimes been interpreted to indicate the existence of a cholinoceptive mechanism along the whole muscle membrane (e.g. Portela *et al.*, 1970) the present results suggest that it would be worth exploring the possibility that these effects may be explicable in terms of chloride ion redistribution.

We enjoyed the help of Dr. D. Colquhoun in some experiments and we are also grateful to Drs. R. Niedergerke, P. Fatt and G. Falk for useful discussions, to Mrs. J. E. Haylett for expert technical assistance and to Mr. F. Ballhatchet for construction and maintenance of apparatus. D. A. T. held an M.R.C. Scholarship during the course of the work.

REFERENCES

ADRIAN, R. H. & FREYGANG, W. H. (1962). The potassium and chloride conductance of frog muscle membrane. *J. Physiol., Lond.*, **163**, 61–103.

ARIËNS, E. J. (1964). *Molecular Pharmacology*, pp. 195–196. New York: Academic Press.

AUERBACH, A. & BETZ, W. (1971). Does curare affect transmitter release? *J. Physiol., Lond.*, **213**, 691–705.

BLUMENTHAL, R. & CHANGEUX, J. P. (1970). About the changes of internal ion concentration in the isolated electroplax during chemical excitation. *Biochim. Biophys. Acta*, **219**, 398–404.

BURNS, B. D. & PATON, W. D. M. (1951). Depolarization of the motor end-plate by decamethonium and acetylcholine. *J. Physiol., Lond.*, **115**, 41–73.

CHANGEUX, J. P. & PODLESKI, T. R. (1968). On the excitability and cooperativity of the electroplax membrane. *Proc. natn. Acad. Sci., U.S.A.*, **59**, 944–950.

CUTHBERT, A. W. & DUNANT, Y. (1970). Diffusion of drugs through stationary water layers as the rate limiting process in their action at membrane receptors. *Br. J. Pharmac.*, **40**, 508–521.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol., Lond.*, **128**, 157–181.

DEL CASTILLO, J. & KATZ, B. (1957). A comparison of acetylcholine and stable depolarizing agents. *Proc. R. Soc. B.*, **146**, 362–368.

FATT, P. (1950). The electromotive action of acetylcholine at the motor end-plate. *J. Physiol., Lond.*, **111**, 408–422.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol., Lond.*, **115**, 320–370.

FELTZ, A. & MALLART, A. (1971a). An analysis of acetylcholine responses of junctional and extrajunctional receptors of frog muscle fibres. *J. Physiol., Lond.*, **218**, 85–100.

FELTZ, A. & MALLART, A. (1971b). Ionic permeability changes induced by some cholinergic agonists on normal and denervated frog muscles. *J. Physiol., Lond.*, **218**, 101–116.

HIGMAN, H. B., PODLESKI, T. R. & BARTELS, E. (1963). Apparent dissociation constants between carbamylcholine, d-tubocurarine and the receptor. *Biochim. biophys. Acta*, **75**, 187–193.

HODGKIN, A. L. (1958). Ionic movements and electrical activity in giant nerve fibres. *Proc. R. Soc. B.*, **148**, 1–37.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol., Lond.*, **148**, 127–160.

HUTTER, O. F. & WARNER, A. E. (1967a). The pH sensitivity of the chloride conductance of frog skeletal muscle. *J. Physiol., Lond.*, **189**, 403–425.

HUTTER, O. F. & WARNER, A. E. (1967b). The effect of pH on the ^{36}Cl efflux from frog skeletal muscle. *J. Physiol., Lond.*, **189**, 427–443.

JENKINSON, D. H. (1960). The antagonism between tubocurarine and substances which depolarize the motor end-plate. *J. Physiol., Lond.*, **152**, 309–324.

KARLIN, A. (1967a). Permeability and internal concentrations of ions during depolarization of the electroplax. *Proc. natn. Acad. Sci. U.S.A.*, **58**, 1162–1167.

KARLIN, A. (1967b). On the application of "a plausible model" of allosteric proteins to the receptor for acetylcholine. *J. theor. Biol.*, **16**, 306–320.

KATZ, B. (1949). Les constantes electriques de la membrane du muscle. *Arch. Sci. physiol.*, **3**, 285–300.

KATZ, B. & THESLEFF, S. (1957). A study of the "desensitization" produced by acetylcholine at the motor end-plate. *J. Physiol., Lond.*, **138**, 63–80.

KATZ, B. & MILEDI, R. (1965). The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc. B.*, **161**, 483–495.

KORDAŠ, M. (1968). The effect of atropine and curarine on the time course of the end-plate potential in frog sartorius muscle. *Int. J. Neuropharmac.*, **7**, 523–530.

MAGAZANIK, L. G. & VYSKOČIL, F. (1970). Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. *J. Physiol., Lond.*, **210**, 507–518.

MANTHEY, A. A. (1966). The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. *J. gen. Physiol.*, **49**, 963–976.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol., Lond.*, **130**, 114–122.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol., Lond.*, **151**, 1–23.

NASTUK, W. L. (1953). Membrane potential changes at a single muscle end-plate produced by transitory application of acetylcholine with an electrically controlled micro-jet. *Fedn Proc.*, **12**, 102.

NASTUK, W. L. & PARSONS, R. L. (1970). Factors in the inactivation of postjunctional membrane receptors of frog skeletal muscle. *J. gen. Physiol.*, **56**, 218–249.

OCHS, S. & MUKHERJEE, A. K. (1959). Action of acetylcholine, choline and d-tubocurarine on the membrane of frog sartorius muscle. *Am. J. Physiol.*, **196**, 1191–1196.

PEPER, K. & MCMAHAN, U. J. (1972). Distribution of acetylcholine receptors in the vicinity of nerve terminals on skeletal muscle of the frog. *Proc. R. Soc. B.*, **181**, 431–440.

PORTELA, A., PEREZ, R. J., VACCARI, J., PEREZ, J. C. & STEWART, P. (1970). Muscle membrane depolarization by acetylcholine, choline and carbamylcholine, near and remote from motor end-plates. *J. Pharmac. exp. Ther.*, **175**, 476–482.

RANG, H. P. (1971). Drug receptors and their function. *Nature, Lond.*, **231**, 91–96.

RANG, H. P. & RITTER, J. M. (1970). On the mechanism of desensitization at cholinergic receptors. *Molec. Pharmacol.*, **6**, 357–382.

RAS, R., DEN HERTOG, A. & LAMMERS, W. (1972). The effect of suxamethonium on the striated muscle fibre outside the end-plate region. *Pflügers Arch. ges. Physiol.*, **333**, 187–196.

TAKEUCHI, A. & TAKEUCHI, N. (1959). Active-phase of frog's end-plate potential. *J. Neurophysiol.*, **22**, 395–411.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of end-plate membrane during the action of transmitter. *J. Physiol., Lond.*, **154**, 52–67.

THESLEFF, S. (1955a). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. *Acta physiol. scand.*, **34**, 218–231.

THESLEFF, S. (1955b). The effects of acetylcholine, decamethonium and succinylcholine on neuromuscular transmission in the rat. *Acta physiol. scand.*, **34**, 386–392.

WERMAN, R. (1969). An electrophysiological approach to drug-receptor mechanisms. *Comp. Biochem. Physiol.*, **30**, 997–1017.

(*Received June* 21, 1972)