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IONIC PROPERTIES OF THE NEUROMUSCULAR JUNCTION OF THE FROG: EFFECTS OF DENERVATION AND pH

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SUMMARY

1. The effects of denervation, reinnervation and pH on the ionic permeability changes mediated by junctional receptors have been studied in muscle fibres of the frog sartorius.

2. The reversal potential of acetylcholine responses in denervated junctions was about 25 mV more negative than in normal junctions.

3. The delay of the change in the ionic properties of junctional receptors was proportional to the nerve stump length: 10 and 14 days for lengths of 12 and 33 mm, respectively.

4. When the motor nerve reinnervates the junction, the reversal potential of the acetylcholine responses comes back to the normal value before the neuromuscular transmission is restored.

5. The Na/K conductance change decreases in high pH solutions. After denervation, the pH profile of this ratio is shifted to the acid values by about two pH units.

6. These observations can be explained by assuming that the Na and K channels differ by the pK of their anionic groups and that the denervation induces an alteration of the sites that bear the charges.

INTRODUCTION

The problem of how the motor nerve regulates some of the properties of the muscle has been a subject of continuing debate. Post-denervation changes in muscle have been attributed to the lack of a 'neural trophic factor' released by the motor endings (Miledi, 1960*a*, 1962). This hypothesis is supported by the observation that the shorter the nerve stump, the earlier the post-denervation changes, viz. fibrillation (Luco & Eyzaguirre, 1955), tetrodotoxin resistant muscle action potentials (Harris &

Thesleff, 1972) decrease in membrane resting potential (Albuquerque, Schuh & Kauffman, 1971).

Another recent set of observations by Vrbová (1970), Drachman & Witzke (1972) and Lømo & Rosenthal (1972) strongly suggest that the excitation of muscle fibres by nerve impulses is the way the nerve controls the extension of the chemosensitivity. However, these results seem in conflict with the observation of a development of chemosensitive areas by muscle injury (Katz & Miledi, 1964) or by a foreign body lying on the muscle surface (Vrbová, 1967; J. del Castillo, personal communication). The supersensitivity observed in muscles where the acetylcholine (ACh) release has been blocked by Botulinum toxin (Thesleff, 1960) or in muscles affected with hereditary 'motor end-plate disease' (Duchen & Stefani, 1971) can also be interpreted as being a consequence of muscle inactivity. On the other hand, the loss in cholinesterase following denervation or botulinum toxin poisoning cannot be attributed to muscle inactivity alone (Guth, Brown & Watson, 1967; Drachman, 1972).

Obviously, the apparent contradictions between the results quoted above must depend, in part, on the nature of the particular post-denervation phenomena under consideration and also on the fact that they are likely the result of the combined action of several causes.

In this paper a new post-denervation phenomenon is described: after the section of the motor nerve and subsequent degeneration of the motor endings, the junctional receptors of frog muscles undergo a sudden change in their ionic properties. It will be seen that the results obtained from experiments where the external pH has been changed may provide a clue for the interpretation of this post-denervation change.

METHODS

Experiments were done in summer or winter frogs (*Rana esculenta* or *R. ridibunda*) stored at room temperature. The sartorius muscle was dissected and immersed in a solution of the following ionic composition (mM): Na, 117·6; K, 2·5; Cl, 121·1; Ca, 1·8; H_2PO_4, 0·4; HPO_4, 1·12. Changes in pH were obtained by adding Tris hydrochloride (5 mM) for alkaline pH values or Tris maleate (5 mM) titrated with NaOH for acid pH values. To avoid the generation of muscle action potentials, tetrodotoxin at a concentration of 5×10^{-8} g/ml. was added to the bath; residual contractions were abolished by disrupting the transverse tubular system using the technique described by Eisenberg, Howell & Vaughan (1971). Muscle fibres treated with this procedure did not show an appreciable loss in resting membrane potential. As shown by Deguchi & Narahashi (1971), in detubulated frog muscles the equilibrium potential for Na^+ and K^+ can be taken as $+50$ and -100 mV, respectively. The muscle was kept at 18–20° C by a continuous flow of the bathing solutions.

A 30–50 MΩ micropipette filled with 2M-ACh was used for iontophoretic application of the drug. The current pulses were of 4 msec duration and of variable intensity; the braking current was about 5×10^{-9} A. The pipettes were pulled either with a

de Fonbrune microforge or with a D. Kopf automatic puller. For pipettes of a given resistance the latter gave tips more suitable for a closer approach to the junctional receptors. Two micro-electrodes filled with 3 M-KCl were inserted 30–80 μm apart into the end-plate area of a sartorius muscle fibre. The electrode used for passing hyper- or depolarizing current pulses had a resistance of 3–5 MΩ; it was connected to a constant current source and the total current was measured with a current-to-voltage converter (see Gage & Eisenberg, 1969).

RESULTS

Identification of junctional spots in denervated muscle

In muscles denervated for periods of time ranging from 1 to 4 weeks or more, the surface of the motor end-plates was explored with a micropipette used for delivering brief electrophoretic ACh pulses. The pipette was moved until a highly sensitive spot was found. The development of connective tissue that invariably occurs in denervated muscles sometimes made the localization of subsynaptic receptors a time consuming procedure. Collagenase (Sigma type III, 0·01 mg/ml.) was occasionally used to soften the connective tissue.

Several criteria have been used to ascertain whether the ACh sensitive spot under consideration really belongs to a former junctional area:

Since junctional spots maintain a high receptor density after denervation (Hartzell & Fambrough, 1972) the sensitivity to electrophoretic ACh pulses (expressed in mV/nC) and the rise time of the responses must be of the same order of magnitude both in normal and in denervated muscles. Moreover, due to the higher receptor density at junctional, as compared to extrajunctional, areas, there is practically no receptor saturation by increasing doses of ACh and the rise time of the junctional responses is independent of the dose (Feltz & Mallart, 1971*a*). No evidence of receptor desensitization that would lead to a lengthening of the rise time of the responses was observed in our experimental conditions due to the relatively high sensitivity of junctional receptors in normal as well as in denervated muscles. In fact, as has been shown previously (Kordas, 1969; Feltz & Mallart, 1971*b*; Magleby & Stevens, 1972*a*) the rise time of the responses is shorter at less polarized membrane potentials due probably to the voltage sensitivity of the transmitter-receptor interaction.

Fig. 1 shows responses to iontophoretic application of ACh to junctional spots from a normal (*A*) and from a denervated (*B*) sartorii from the same frog, placed side by side in the same recording chamber. Responses from either muscle were quite similar in respect to receptor sensitivity and rise time. Both parameters may vary from one end-plate to another due to differences in the positioning of the ACh pipette and on the shape of its tip.

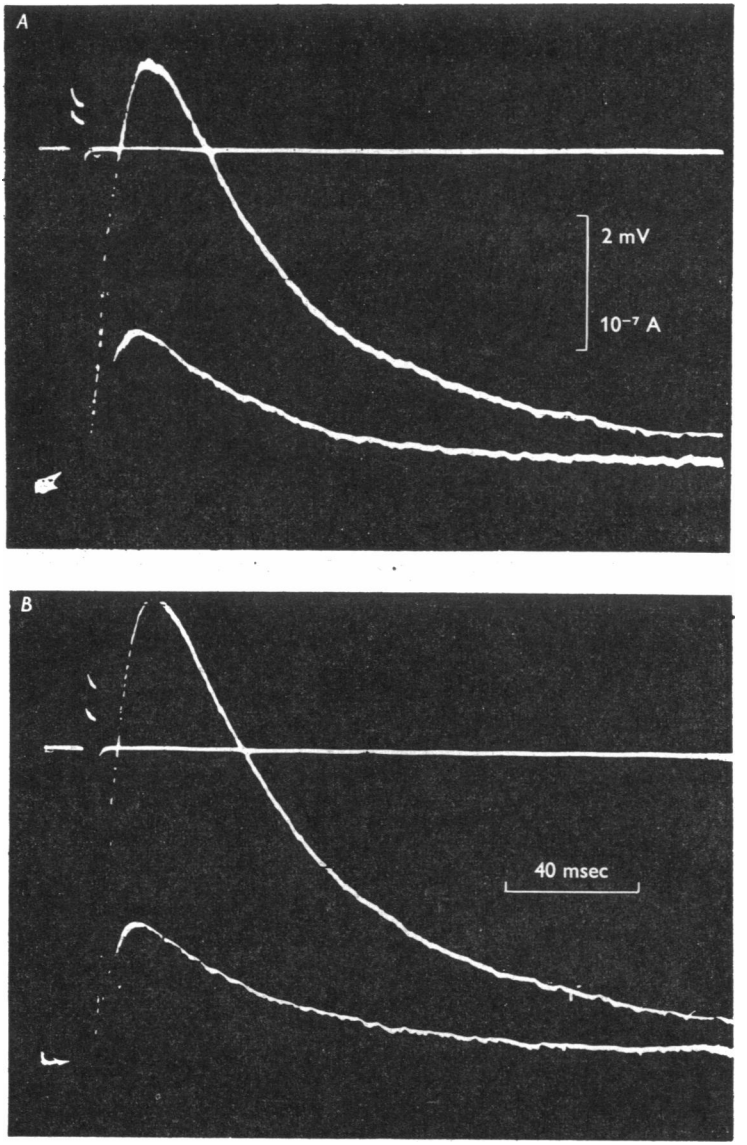

Fig. 1. Responses to iontophoretic ACh elicited at junctional spots from an intact innervated (*A*) and a 21-day denervated sartorii (*B*) from the same frog, recorded in the same experimental conditions. The reversal potential was −14 mV in the former and −43 mV in the latter. Same time, voltage and current calibrations for *A* and *B*.

Sensitivities ranging from 50 to 300 mV/nC and rise times from 5 to 15 msec were found in normal as well as in denervated junctions.

A further test that has occasionally been used is the potentiating effect of the edrophonium (a fast acting anticholinesterase) on the ACh response

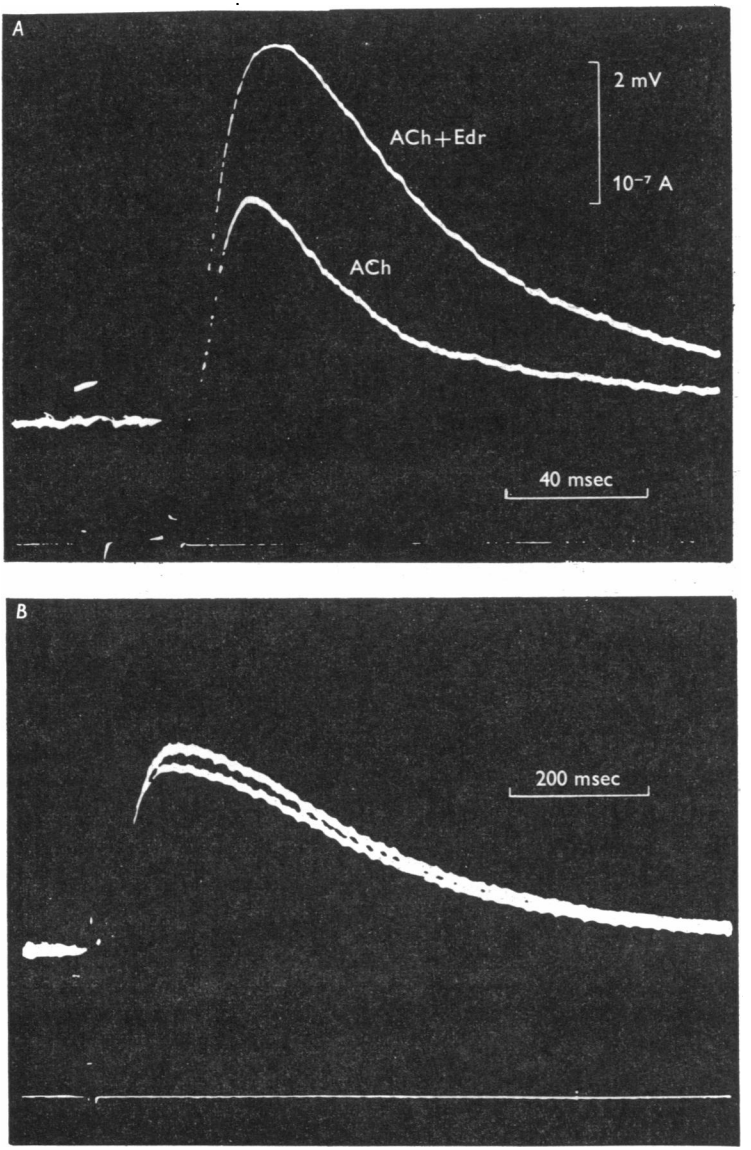

Fig. 2. Potentiation of the ACh responses by a preceding pulse of edrophonium. *A*, Junctional spot from a 18-day denervated junction; *B*, extrajunctional spot from the same muscle fibre. Same voltage and current calibration for *A* and *B*.

when both drugs are delivered on a membrane containing cholinesterase (Katz & Thesleff, 1957). In the experiment illustrated in Fig. 2 a twin barrel pipette with one of its channels filled with edrophonium and the other with ACh has been used. Since cholinesterase activity is much higher at junctional than at extrajunctional sites, and a substantial amount is maintained after denervation (Guth, Albers & Brown, 1964), the junctional responses of denervated muscle are expected to be enhanced when the edrophonium precedes the ACh pulse whereas the extrajunctional responses should show little or no change.

The reversal potential of ACh responses from denervated motor end-plates

In a series of experiments, the sartorius muscle was denervated by sectioning the sciatic nerve at the pelvic level and the innervation prevented by removing a length of several millimetres of nerve. It was observed that for periods of denervation longer than about 15 days, the ACh responses mediated by the junctional receptors reverse at a membrane potential of about -42 mV instead of -15 mV as in normal junctions.

In Fig. 5, the amplitude of the ACh response elicited at a denervated junctional spot is plotted as a function of the membrane potential. Response amplitude is zero at a membrane potential of -41 mV. The mean value of the reversal potential (E_R) obtained in sixty-one denervated junctions by extrapolation from the amplitudes measured at membrane potentials ranging from -120 to -50 mV was $-41{\cdot}3 \pm 4$/mV (S.D.). This value is similar to that obtained for responses mediated by extrajunctional receptors of both normal and denervated muscle fibres (Feltz & Mallart, 1971*b*).

In several instances, we were able to depolarize the membrane beyond the reversal potential of the responses. In this case the null point was about 10 mV more positive than that obtained by extrapolation. Thus a non-linearity frequently exists in the membrane potential-response amplitude relationship, the inflexion point being at a membrane potential around -50 mV. This non-linearity was more evident in those preparations where the values of E_R obtained by extrapolation were between -10 and -20 mV than in those where the extrapolated E_R fell between -40 and -50 mV. The reason is that in the latter cases the inflexion point of the membrane potential–amplitude relationship is already very near the reversal potential of the responses. This phenomenon has been already observed in earlier experiments (Feltz & Mallart, 1971*b*), both for junctional and extrajunctional responses. Our estimation of E_R by actual reversal of responses in normal junctions agrees with the values of 0 to -10 mV obtained by several authors for the reversal of the end-plate potential in voltage clamped preparations (Kordas, 1970, 1972; Deguchi

& Narahashi, 1971; Magleby & Stevens, 1972*b*). On the other hand, our extrapolated reversal potential of -10 to -20 mV corresponds to that found by Takeuchi & Takeuchi (1960) by voltage clamp and extrapolation.

Effect of nerve stump length on the timing of the membrane changes

The E_R of the ACh responses was measured after different periods of denervation. During the early days that follow the section of the motor nerve no change in the value of E_R was observed. Then for a period of 2–3 days, an increasing number of neuromuscular junctions can be found in which a change in E_R has occurred. In a given muscle it is possible to find junctions whose ACh responses reverse at about -16 mV (as in normals) while in other fibres junctional responses reverse at about -40 mV.

During the later stages of denervation only responses with a modal value of E_R of -42 mV could be obtained from all the explored end-plates either at junctional or at extrajunctional chemosensitive areas.

It has been observed that the timing of the change in the ionic properties of the synaptic membrane depends on the level of the nerve section: the longer the nerve stump, the longer the delay of the shift from the normal to the denervated type of permeability.

The sartorius muscle was denervated either by sectioning the sciatic nerve at the pelvic level or the sartorius nerve midway between the point where it leaves its parent branch and the muscle. A small length of nerve was removed in order to prevent reinnervation. The nerve stump length was about 30–35 mm in the former and 10–13 mm in the latter type of denervation.

The reversal potential of the responses to iontophoretic application of ACh to junctional receptors was measured in both experimental conditions at different times after nerve section. The results of these experiments are displayed in Fig. 3 where the values of E_R obtained at individual end-plates from both 'long' (open circles) and 'short' (filled circles) denervated muscles are plotted as a function of time of denervation. As can be seen, the normal ionic properties of the junctional receptors are maintained for about 10 days after a 'short' nerve section and for about 14 days after a 'long' section of the motor nerve. In other words, the shift of E_R of the ACh responses from -16 to -42 mV is delayed by about 4 days when the nerve stump is 20 mm longer. No significant differences between summer and winter frogs were observed in the timing of the shift of E_R.

By the time the shift in E_R occurs, the neuromuscular transmission has already failed as can be inferred from the absence of spontaneous activity. Although in the present experiments no attempt to establish a precise timing of neuromuscular failure was done, some correlation between both

events may be obtained using the data of Harris & Miledi (1972). We can estimate approximatively that the transmission failed about 2–3 days before the change in the ionic properties of the junctional receptors.

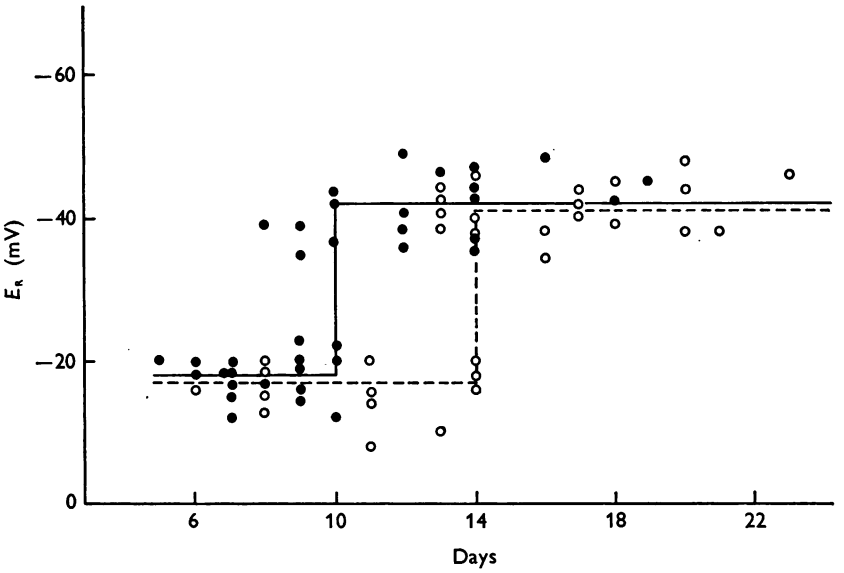

Fig. 3. Individual values of the reversal potential of ACh responses elicited at junctional spots, plotted as a function of the time of denervation. Dots represent short nerve stump and open circles long nerve stump.

Reinnervation of the neuromuscular junction

The sartorius muscle was denervated by crushing its nerve midway between its origin in the branch from the sciatic and the muscle. In this way the time taken by the regenerating axons to reach the end-plates is shorter than if the nerve was transected.

Approximately 3–4 weeks after the crushing, the re-establishment of the neuromuscular transmission was checked. While in some junctions an e.p.p. was present on stimulation of the motor nerve, in others no evoked activity could be elicited. When present, the spontaneous activity consisted of slow miniature potentials like that of denervated muscle but with a higher frequency (Miledi, 1960*b*; Dennis & Miledi, 1971). One example is shown in Fig. 4*B*.

Occasionally it was observed that the newly reinnervated neuromuscular junctions respond by a composite e.p.p. (Fig. 4*A*). This fractionation of the e.p.p. may result either from a local blockade of the motor nerve terminals (Katz & Miledi, 1968) or from a multiple innervation of the end-plate. Regenerating motor axons have a slower conduction velocity

(Miledi, 1960*b*) and one may wonder whether they have also a lower safety factor for the propagation of nerve impulses through the terminal branchings. On the other hand, one may consider a multiple innervation of the end-plate as a more plausible explanation. Such a multiple innervation has been observed in new-born rat muscles (Redfern, 1970) and in adult rat regenerating junctions (Saito & Zacks, 1969). Obviously it represents a rare and transient feature of the early stages of end-plate reinnervation. Later, one axon takes over and the end-plate becomes single innervated as is usual in normal skeletal twitch muscle.

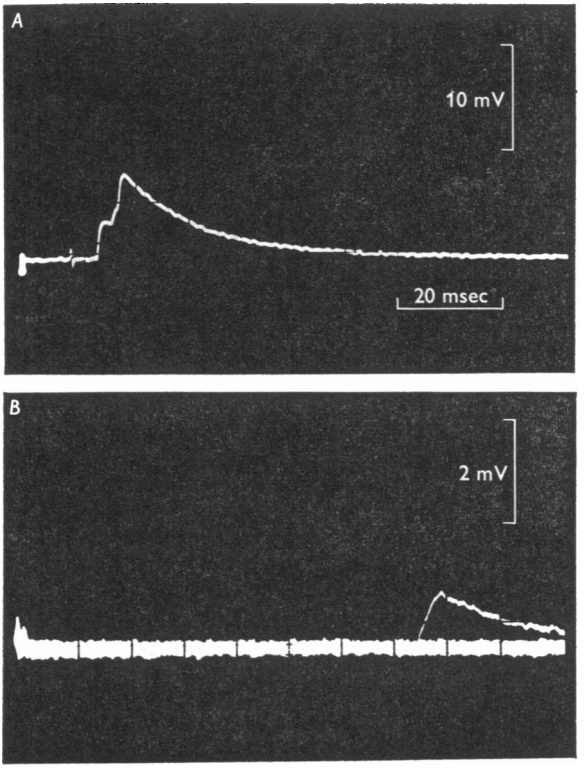

Fig. 4. A composite end-plate potential recorded at a regenerating junction: its shape did not vary with successive trials. *B*, spontaneous miniature potential from a regenerating junction. In both junctions the value of the reversal potential of the ACh responses was like in normal junctions. Same time calibration for *A* and *B*.

The reversal potential of the responses mediated by junctional receptors has been measured in muscles undergoing reinnervation. It was observed that about 25–35 days after crushing the motor nerve 12 mm from the muscle, the value of the reversal potential of the responses to

iontophoretic ACh pulses has returned to that usually found in normal junctions. This was true, not only for normally transmitting junctions, but also for those in which no end-plate potentials could be evoked and where a denervated type of spontaneous miniature activity was present. In other words, the return of the junctional receptors to their normal ionic properties occurs before the re-establishment of neuromuscular transmission.

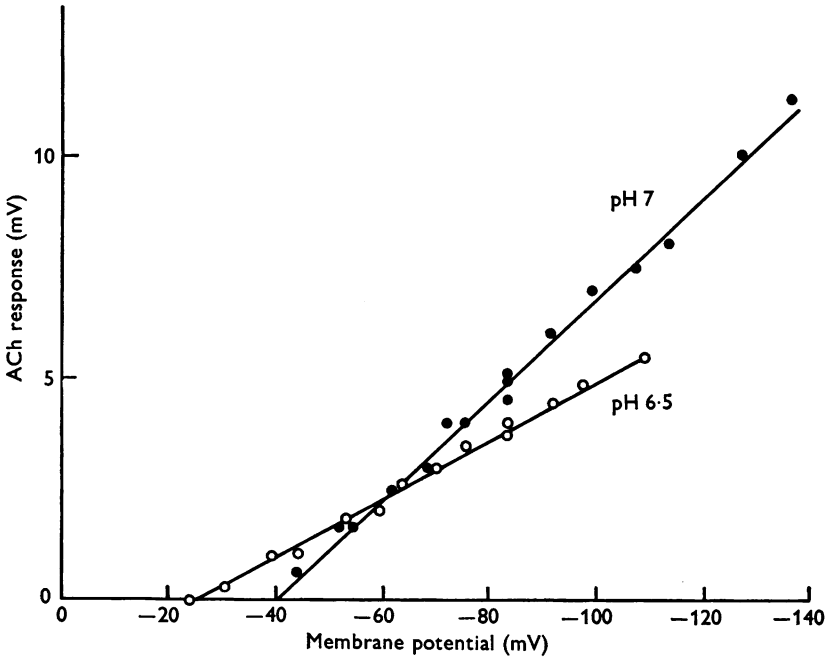

Fig. 5. Relationship between ACh response amplitude and membrane potential in a 24-day denervated junction measured at pH 7 and pH 6·5. Due probably to a slight displacement of the ACh pipette while changing fluids, response amplitude is smaller in the pH 6·5 solution. The resting potential was −84 mV.

In a control series of experiments the reinnervation was prevented by removing 3–4 mm of nerve. In this case, for comparable times of denervation and nerve stump length the reversal potential of the responses was always that of a denervated junction. In this way one can rule out the possibility of a spontaneous return to the normal ionic properties as an explanation for the results reported above. In another control series, the muscles were examined at shorter periods of time after crushing the nerve; the reversal potential of the responses was always like that in muscles kept denervated by nerve section for comparable lengths of time.

Effect of changes in external pH

In a previous study (Feltz & Mallart, 1971*b*) it was suggested that the observed differences between the ionic properties of the junctional and extrajunctional receptors may depend on the field strength of the negative charges of the ionic pathways associated to either type of receptor. An obvious way to test this hypothesis is to modify the electric field strength of the ionic binding sites by changes in the external pH.

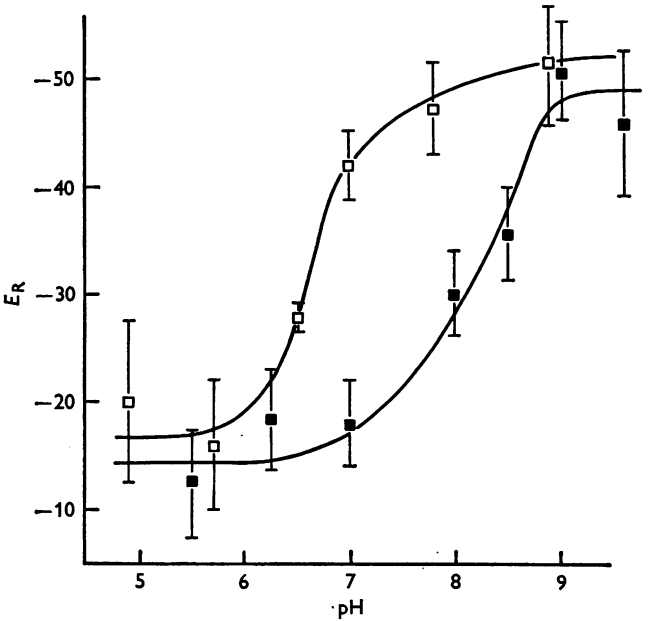

Fig. 6. Dependence of the value of the reversal potential of ACh responses of the pH of the external medium. The mean values of E_R from normal and denervated muscles are represented by black and white squares, respectively. The bars indicate the S.D. of the mean of 8 to 10 observations.

Normal or denervated muscles were bathed in solutions buffered with 5 mM Tris at different pH ranging from 5 to 9·5. Several sets of measurements of the reversal potential of ACh responses from different junctions were taken at the selected pH. Attempts to measure in the same muscle the effects of different solutions were impractical because of the long time required to obtain a complete recovery, especially when the effect of extreme pH values was tested. Fig. 5 shows the relationship between membrane potential and amplitude of responses obtained in two junctional spots from a denervated muscle, bathed in solutions of pH 6·5 and pH 7.

The results of this series of experiments are summarized in Fig. 6 where

the E_R of the responses is plotted as a function of the external pH for normal (filled squares) and denervated muscles (open squares). As can be seen, in normals low pH has little effect on the ionic properties of the receptors while in high pH the responses reverse at a membrane potential near -50 mV, i.e. a value close to that of denervated muscles in normal or alkaline medium. On the contrary, in denervated muscles an alkaline pH induces only a moderate change whereas in acid pH the responses reverse at about -15 mV like that of normal muscles in neutral or acid external solutions. One may regard the E_R–pH relationship as an acid-base titration curve: the structures that mediate the ionic permeabilities both in normal and in denervated junctions behave in acid pH as proton donors and in alkaline pH as proton acceptors. In this respect, the main difference between normal and denervated muscles would be a shift in the apparent dissociation constant K of the reaction: pK = 8·2 in the former and = 6·6 in the latter.

DISCUSSION

There are two main findings in this study. First, a change in the ionic properties of the synaptic membrane after motor nerve degeneration and the return to the normal type of permeability as the regenerating nerve grows into the old junction. Secondly, changes in the external pH can mimic, to a certain extent, the effects of denervation and reinnervation.

The present results exclude the action of muscle inactivity as a cause of the change in the ionic properties of junctional receptors, first, because the nerve stump length has an effect on the time of appearance of the change, and, secondly, because the normal type of permeability comes back before the re-establishment of neuromuscular transmission. Probably the presence of the nerve endings over the post-synaptic membrane induces, by a not yet well understood mechanism, a local alteration of the structures that control the ionic movements across the membrane. This alteration would lead to the 'normal' type of synaptic permeability. It is likely that the factor responsible for the post-synaptic change depends on a specific neural action, since the presence of the Schwann cell in the synaptic gutter cannot compensate for the loss of synaptic contact between nerve and muscle.

Since on morphological grounds the denervated junction can be regarded as a non-innervated chemosensitive membrane, one would expect that its ionic properties are like those of extrajunctional receptive areas. In fact, the present results show that the reversal potential of ACh responses mediated by junctional receptors of denervated muscle is similar to that observed by Feltz & Mallart (1971*b*) at extrajunctional areas of

normal or denervated muscles. These authors have shown that the shift in the reversal potential from -15 to -45 mV corresponds to a change in the ratio of the increase of the synaptic conductances $\Delta g_{Na}/\Delta g_K$ from 1·29 to 0·60.

The results reported in this paper suggest that the ionic pathways of the chemosensitive membrane can adopt two states which are defined by their ratio of conductance increase to Na^+ and K^+: $\Delta g_{Na}/\Delta g_K = 1{\cdot}29$ and $\Delta g_{Na}/\Delta g_K = 0{\cdot}60$; variations in pH from 5 to 9 induce a progressive shift from the first state to the second. At pH 7, after muscle denervation or reinnervation, the membrane jumps from one state to the other.

These observations can be explained by assuming that the pathways for Na^+ and K^+ are independent and that they bear negative charges. Convincing evidence for separate Na^+ and K^+ synaptic conductances can be drawn from the experiments on the frog neuromuscular junction with high extracellular Ca^{2+} (Takeuchi, 1963) in which the Na^+ conductance alone is reduced, and from experiments with local anaesthetics (Maeno, 1966; Maeno, Edwards & Hashimura, 1971; Deguchi & Narahashi, 1971; see, however, Kordaš, 1970) that affect differentially both conductances.

The postulate of negatively charged sites in both ionic channels seems to be required to explain the effect of changes in external pH on the variation of the ratio $\Delta g_{Na}/\Delta g_K$ shown by the present investigations.

It can be assumed that the charged sites of the ionic pathways can bind either Na^+ or K^+ and that the magnitude of the ionic fluxes depends on the amount of site-cation complex formed, which in turn would depend both on the cationic concentrations and on the number of sites in the dissociated (anionic) form. The fact that both K^+ and Na^+ are inequally distributed on either side of the membrane is irrelevant for the present discussion since inward or outward synaptic fluxes for each ion are very small compared to their outside or inside concentrations. In this situation the limiting factor would be the number of active groups in the dissociated form. For each channel, the degree of ionization of the sites would be dependent on their pK and on the pH of the surrounding medium. Thus, in high pH both Δg_K and Δg_{Na} are expected to increase, but, since the present results indicate that $\Delta g_{Na}/\Delta g_K$ actually decreases, it can be concluded that, in high pH, the increase of the Na conductance must be relatively lower than that of the K conductance. Differences in the pK of the two ionic pathways can explain this situation. For instance, one can imagine that the pK of the anionic sites of the K channels is in the range of the pH values explored whereas that of Na sites lies outside this range, in such a way that Δg_{Na} is little affected by the pH changes used in this study.

After muscle denervation, the pH profile of the conductance change at

junctional receptors shifts towards the acid side by about 2 pH units. One can admit that the pH dependence of the reaction of Na^+ and K^+ with the anionic sites of their channels is characteristic of some group, or interacting groups, involved in the reactions of the site-cation complex. Consequently, it can be speculated that the presence of the motor endings on the subsynaptic membrane may induce an alteration of molecular structure of the sites that bear the active groups of the channels, resulting in a shift of the pK of the latter to the alkaline side.

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