J. Physiol. (1979), **295**, 273–281
With 5 text-figures
Printed in Great Britain

IONIC BASIS FOR ELECTRICAL PROPERTIES OF TONIC FIBRES IN RAT EXTRAOCULAR MUSCLES

BY ARDITH Y. BONDI AND D. J. CHIARANDINI

From the Departments of Ophthalmology and Physiology and Biophysics, New York University Medical Center, New York, N.Y. 10016, U.S.A.

(*Received 7 February 1979*)

SUMMARY

1. The ionic conductances underlying some of the electrophysiological properties of multiply innervated or tonic fibres of rat extraocular muscles were examined *in vitro* with double-barrelled micro-electrodes.

2. Exposure of the muscle to a Cl-free saline did not change the effective resistance (R_{eff}) of tonic fibres which was $5{\cdot}14 \pm 0{\cdot}45$ MΩ ($n = 7$) in control saline and $4{\cdot}78 \pm 0{\cdot}45$ MΩ ($n = 12$) in Cl-free saline ($P > 0{\cdot}1$). In contrast, in singly innervated or twitch fibres Cl removal increased R_{eff} from $1{\cdot}77 \pm 0{\cdot}21$ MΩ ($n = 19$) to $2{\cdot}69 \pm 0{\cdot}12$ MΩ ($n = 22$) ($P < 0{\cdot}001$).

3. Tonic fibres with membrane potentials restored to -80 mV by injecting current responded to intracellular depolarizing pulses with a brief, slow response (slow peak potential) which added to the rising phase of the electrotonic potential. The slow peak potential began at a membrane potential of -40 to -35 mV and was graded. Increasing depolarizations evoked faster and larger responses which did not overshoot the zero level of membrane potential.

4. The slow peak potential was not blocked by 10 μM-D-600 hydrochloride but was markedly reduced by the absence of Na and by 10 μM-tetrodotoxin. The response was broadened about five times by 25 mM-tetraethylammonium.

5. Raising bath temperature from 21–25 °C to 37 °C reversibly depressed and shortened the slow peak potential but did not transform it into an action potential.

6. It is concluded that the characteristic high R_{eff} of tonic fibres results from a lack of a membrane conductance to Cl and that the slow peak potential involves the transient activation of Na and K channels which are pharmacologically similar to the respective channels of twitch fibres.

INTRODUCTION

Previous electrophysiological work (Hess & Pilar, 1963; Matyushkin, 1964) has demonstrated that mammalian extraocular muscles contain singly and multiply innervated muscle fibres. The first type is equivalent to twitch fibres of other mammalian muscles and comprises most of the muscle cells. The second type is physiologically similar to slow or tonic fibres of frog (Hess & Pilar, 1963; Matyushkin, 1964; Chiarandini, 1976) and is not found in other muscles of mammals with the

0022-3751/79/5430-0016 $01.50

exception of the ear muscles (Fernand & Hess, 1969) and oesophagus musculature (Floyd, 1973).

The electrical properties and synaptic activity of the multiply innervated or tonic fibres of rat extraocular muscles have been examined recently by Chiarandini & Stefani (1979). These authors demonstrated that these fibres exhibit a multifocal spontaneous synaptic activity, have a very high effective resistance and a large time constant and, with resting potentials restored to physiological levels, lack action potentials. These fibres, however, responded to depolarizing stimuli with brief, graded depolarizations considered to be 'local' responses.

The present investigation was undertaken to analyse the ionic conductances underlying some of these electrical properties. These results have been reported in part (Bondi & Chiarandini, 1978).

METHODS

Inferior rectus muscles were used from Wistar rats weighing 125–175 g. The animals were anaesthetized with sodium thiamylal (5 mg/100 g wt.). The muscle was isolated from the animal with its proximal end attached to a fragment of the presphenoid bone and with its distal end to the sclera and placed in a chamber with a volume of 3 ml. The muscle was oxygenated continually during the dissection. Conventional intracellular techniques were used to record from and inject current into cells using double-barrelled micro-electrodes. The recording barrel was filled with 3 M-KCl and had a resistance of 30–50 MΩ. Very small micro-electrode tips were required to minimize injury to the cells which have a diameter of only 15–40 μM (Mayr, 1971). The other barrel was filled with 1·5 M-K citrate. Coupling resistance between the two barrels was usually about 150 kΩ and never more than 300 kΩ.

Rectangular pulses of current with a duration of 130–180 msec were delivered to the fibres between the micro-electrode and a Ag–AgCl virtual ground electrode in the bath. In all fibres, DC current also was applied to maintain intracellular membrane potentials of −80 mV unless otherwise specified. Injected current was measured with an operational amplifier in the ammeter configuration connected with a Ag–AgCl wire to the bath. The effective resistance (R_{eff}) of the cells was calculated, by dividing the voltage deflexion produced by small pulses of hyperpolarizing current by the intensity of such current. All recordings were made from fibres impaled in the superficial layers of the distal third of the global surface of the muscles.

Muscles were bathed in a saline containing (mM): NaCl, 136; KCl, 5; $CaCl_2$, 10; $MgSO_4$, 1·2; glucose, 11; and imidazole sulphate, 5. The relatively high Ca concentration helped to reduce the extent of membrane damage inflicted by the double-barrelled micro-electrodes. Cl-free saline was made up by substituting the chloride salts by appropriate salts prepared by neutralizing methanesulphonic acid (Eastman Organic Chemicals, Rochester, N.Y.) with NaOH or KOH, or by reacting it with $CaCO_3$. Methanesulphonate was selected as a Cl substitute because it has been found to affect Ca^{2+} activity minimally in a similar physiological saline (Kenyon & Gibbons, 1977) and the elevated extracellular Ca^{2+} concentration could be thus maintained. Na-free saline was prepared by substituting an equimolar concentration of tris(hydroxymethyl)-aminomethane (TRIS) for the NaCl. Solutions containing tetrodotoxin (TTX), D-600 hydrochloride (D-600) and tetraethylammonium (TEA) were prepared by adding the necessary amounts of these drugs to control saline. D-600 hydrochloride was a gift from Knoll AG, Chemische Fabriken, Ludwigshafen am Rhein, Germany. All solutions had a pH of 7·35, were oxygenated continually and flowed through the muscle chamber at a rate of about 1·5 ml./min. Experiments were carried out at room temperature (21–25 °C) except when otherwise specified. Results are expressed as mean ± S.E. of mean. Some of the records included in the Figures were reinforced.

RESULTS

Resting Cl permeability. Multiply innervated or tonic fibres were distinguished from singly innervated or twitch fibres by their characteristic spontaneous synaptic activity and by the long time constant of their membrane (12–50 msec) and high R_{eff} (3·3–9·6 MΩ) following criteria used by Chiarandini & Stefani (1979). The resting potential of tonic fibres pentrated with double-barreled micro-electrodes ranged from −24 to −70 mV. To enable the comparison of results, cells were systematically hyperpolarized to −80 mV before further testing.

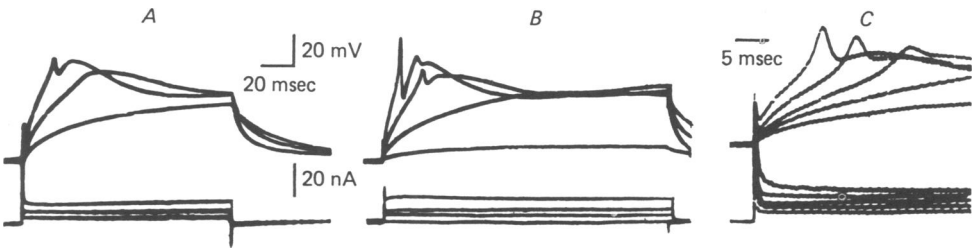

Fig. 1. Variation of the amplitude of slow peak potentials. Records obtained in fibres from three different muscles. Cells were hyperpolarized to −80 mV by injecting current intracellularly. Upper traces: voltage; lower traces: applied current. Most of the responses resemble record *A*. Very few responses were as large as *B*, and in no case did the slow peak potentials exceed the 0 mV level of membrane potential. The graded nature of the response is clearly seen in record *C*, obtained at a four times faster sweep speed.

It has been suggested (Chiarandini & Stefani, 1979) that the long time constant and high R_{eff} of tonic fibres might be a consequence of a low or absent membrane permability to Cl. This possibility was tested by comparing the R_{eff} of tonic and twitch fibres in five inferior rectus muscles before and after perfusion with Cl-free saline. Attempts to make measurements on the same fibres before and after treatment were unsuccessful because Cl-free solution evoked spontaneous twitching which in most cases dislodged the micro-electrode. Therefore, in each muscle two groups of muscle fibres were sampled, one bathed in control saline and the other exposed to Cl-free saline for about 15–90 min. Cl removal did not modify significantly the R_{eff} of tonic fibres which was $5{\cdot}14 \pm 0{\cdot}45$ MΩ ($n = 7$) in control saline and $4{\cdot}78 \pm 0{\cdot}45$ MΩ ($n = 12$) in Cl-free saline ($P > 0{\cdot}1$). In contrast, the same procedure caused an expected increase of the R_{eff} of twitch fibres from $1{\cdot}77 \pm 0{\cdot}21$ MΩ ($n = 19$) in control saline to $2{\cdot}79 \pm 0{\cdot}12$ MΩ ($n = 22$) in Cl-free saline ($P < 0{\cdot}001$).

Responses to depolarizing pulses. As reported previously (Chiarandini & Stefani, 1979) depolarizing pulses in a wide range of amplitudes did not trigger all-or-none action potentials in multiply innervated fibres. Instead, when the membrane potential of the cell reached −40 to −35 mV the pulses evoked a brief, depolarizing response which added to the rising phase of the passive electrotonic response. The brief response, to which we shall refer as the slow peak potential, consisted of an upstroke followed by a rapid falling phase which reversed abruptly defining a notch. This was followed by a further depolarization which declined slowly to a plateau. The con-

figuration of the slow peak potential was very characteristic of this type of cell. Fig. 1 illustrates this response in three different fibres. The duration of the slow peak potential ranged from 5 to 7 msec as measured at its base. Slow peak potentials could not be evoked with anode break stimulation.

The slow peak potential was graded, becoming larger and faster when increasing levels of depolarization were applied to a given fibre. This is shown in Fig. 1, particularly in *C* obtained at a fast sweep speed. The amplitude of the slow peak potential for a given depolarization varied markedly from fibre to fibre (Fig. 1*A* and *B*). The extreme range of variation was from about 1 mV to as much as 38 mV in an unusual cell (Fig. 1*B*). The majority of the fibres produced responses which most closely resemble Fig. 1*A*. There was no obvious correlation between the amplitude of slow peak potentials evoked by a given depolarization and the initial resting potential or R_{eff}. The slow peak potential never reached the zero level of membrane potential. In a total of fifty-eight control tonic fibres examined, no response was recorded which resembled an action potential in amplitude and time course.

The variability of the responses among fibres of different muscles made their quantitative comparison very difficult. However, the time course and amplitude of slow peak potentials in fibres of the same muscle tended to be rather uniform. Therefore the results reported in the following sections include only observations made before and after a given treatment either in different fibres of the same muscle or in the same fibre. The number of experiments in which this last sort of experiment was successful was rather small because they required that the same fibre be held for at least 20 min without appreciable loss of resting potential, which was rarely possible.

Effect of temperature on the slow peak response. Experiments in cat extraocular muscles (Hess & Pilar, 1963) have demonstrated that multiply innervated fibres do not produce action potentials. However, it has been argued (Bach-y-Rita, 1971) that these experiments were performed at room temperature at which, hypothetically, the spike mechanism could be inactivated. This criticism can be applied equally to previous work in rat (Chiarandini & Stefani, 1979) and to the present study. Therefore we studied in three fibres the effects of raising the bath temperature from 21–25 °C to 37 °C on the configuration of the slow peak potential. It was found that the response was shortened in duration and had a somewhat smaller amplitude. In no case a transformation into an action potential was observed. The effect of increased temperature was reversed when the muscle was returned to room temperature.

Effect of D-600. To determine if an increase in Ca conductance is involved in the slow peak potential of tonic fibres, six muscles were treated with a concentration of D-600 (10^{-5} M) known to antagonize Ca conductance but not likely to have significant local anesthetic activity or a Ca-releasing effect (Dörrscheidt-Käfer, 1977). In five multiply innervated fibres the response to depolarizing pulses before and after adding D-600 was studied. D-600 did not depress the slow peak potentials even after a 40 min exposure. Furthermore, six other tonic fibres impaled between 15 and 60 min after adding D-600 exhibited slow peak potentials comparable to those recorded in other fibres of the same muscle under control conditions. Fig. 2 illustrates the responses of three fibres from the same muscle, one in control saline (Fig. 2*A*) and the other two, 45 and 60 min in D-600 (Figs. 2*B* and *C*).

Effect of TTX and Na removal. The apparent lack of Ca involvement in the slow peak potential implied that Na should play a major role in this phenomenon. Fig. 3 demonstrates the depressant effect of TTX. Records in Figs. 3*A* and *B* were obtained from the same fibre which was tested before (*A*) and after 20 min in 1·25 μM-TTX (*B*). Although the peak potential appeared absent in *B*, it was subsequently found in other fibres that by increasing the stimulus intensity, small, rounded responses could

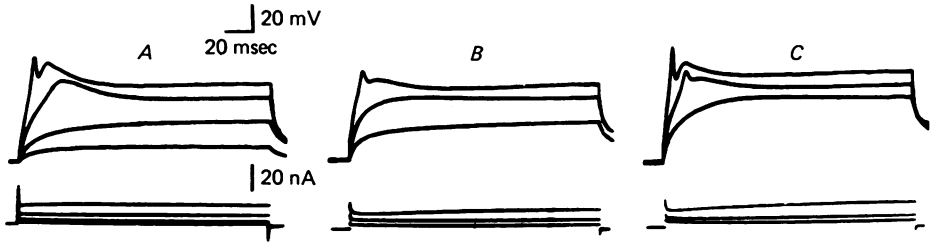

Fig. 2. Effect of D-600 hydrochloride. Records obtained in the same muscle. *A*: control response. *B* and *C*: responses obtained in two other fibres 45 and 60 min after perfusing with D-600. The slow peak potential was not blocked.

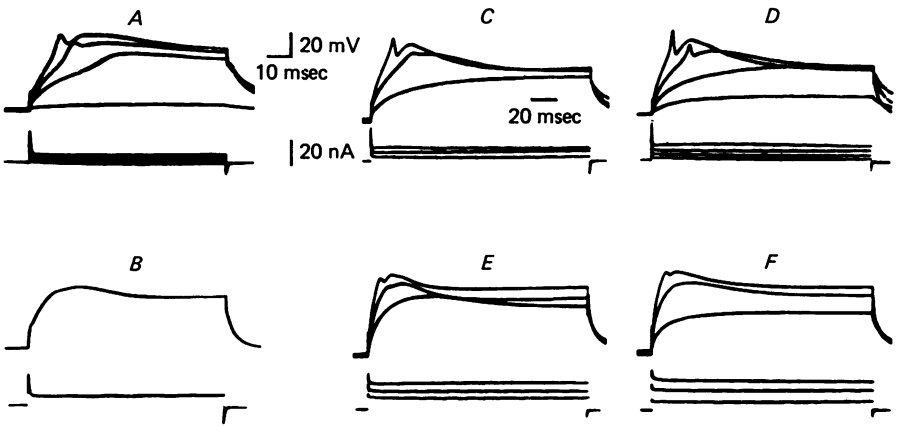

Fig. 3. Effects of tetrodotoxin on slow peak potentials. *A*: control response. *B*: the response of the same fibre was completely blocked 20 min after adding 1·25 μM-TTX. *C* and *D*: control responses in two different fibres of another muscle. *E* and *F*: the same muscle as *C* and *D*, records obtained 67 min (*E*) and 72 min (*F*) after starting perfusions with 10 μM-TTX. Note that the largest pulses evoked small, rounded responses.

be elicited even if exposed to a very high concentration of TTX such as 10 μM. This observation is illustrated in the records of Fig. 3*E* and *F* which were obtained from two other fibres in a muscle perfused with 10 μM-TTX for respectively 67 and 72 min. Control records for the same fibres are not available but Figs. 3*C* and *D* show control responses from two other fibres in the same muscle, before it was perfused with TTX. Similar results were obtained in thirteen fibres total.

To investigate further the participation of Na in the slow peak potential, the effect of a Na-free solution was studied in another group of muscles. The removal of Na was followed by a large hyperpolarization of the fibres which amounted to 10–15 mV and by an increase in the frequency and amplitude of the spontaneous

synaptic potentials. These, which normally range in amplitude from 0·4 to 5 mV, could occasionally be as large as 10–15 mV. This burst of activity started to decline 8–10 min after the change of solution and by 15 min spontaneous synaptic potentials had usually disappeared.

Figs. 4*A* and *B* are records from one fibre tested before and 18 min after Na removal. In the absence of Na the slow peak potential disappeared completely. In

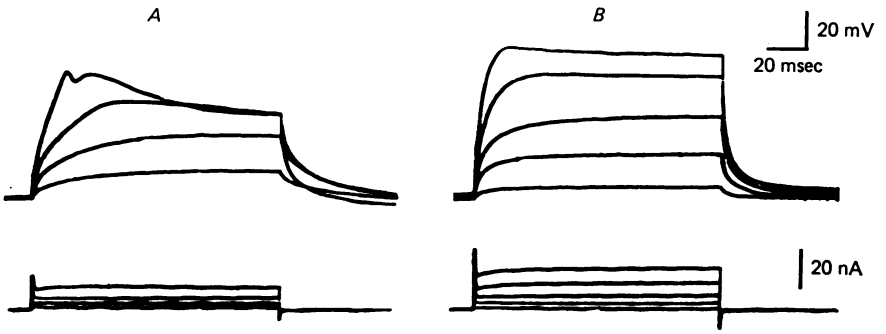

Fig. 4. Effect of Na removal on the slow peak potential. Records obtained in the same fibre. *A*: control response *B*: the response was completely abolished 18 min after exposing the muscle to Na-free TRIS saline. Note that the delayed rectification present in the two top traces of *A* became evident only at a larger level of depolarization after perfusion with Na-free saline.

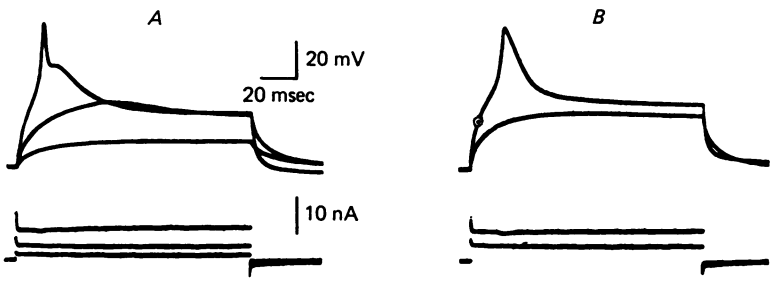

Fig. 5. Effect of tetraethylammonium. Records obtained in the same fibre. *A*: control response. *B*: 11 min after exposure to 25 mM-TEA. The duration of the slow peak potential increased 3–4 times in this cell and the notch of the falling phase disappeared. The level of polarization reached at the end of the pulse increased indicating a reduction of K-delayed rectification.

contrast with the small, rounded responses evoked by large pulses in the presence of TTX (Figs. 3*E* and *F*), in the absence of Na responses could not be produced with any intensity of current.

In the course of these experiments it was also observed that in the Na-free TRIS saline, delayed rectification became less evident in all fibres studied (compare Figs. 4*A* and *B*).

Effect of tetraethylammonium. The presence of delayed rectification in tonic fibres of rat extraocular muscles has been described by Chiarandini & Stefani (1979). To determine whether this voltage-dependent conductance participates in the repolar-

ization phase of the slow peak potential, the effects of TEA, a known blocker of the delayed rectifier (Stanfield, 1970), on the time course of this response were investigated. One of these experiments is illustrated in Fig. 5 which shows for the same fibre a control response (*A*) and a response obtained 11 min after perfusion with 25 mM-TEA (*B*). TEA did not affect the upstroke of the slow peak potential but markedly increased the duration of the peak about five times so that the notch portion of the response disappeared. Similar results were obtained in seven fibres.

A few fibres which had been treated with TEA were subsequently exposed to 10 μM-TTX and the response to large depolarizing pulses was tested. In no case were there observed the small, rounded responses such as those recorded in the presence of TTX alone (Fig. 3*E* and *F*).

DISCUSSION

This study confirms previous observations (Hess & Pilar, 1963; Matyushkin, 1964; Chiarandini & Stefani, 1979) that multiply innervated or tonic fibres of extraocular muscles do not respond to intracellular stimulation with all-or-none action potentials. The slow peak potentials observed upon application of depolarizing pulses cannot be interpreted as abortive action potentials resulting from cathodal depression of the cells, since the fibres were uniformly hyperpolarized to -80 mV. Also, slow peak potentials were the only response that could be evoked in fibres having spontaneous resting potentials of about -70 mV. Furthermore, near normal action potentials could be evoked in twitch fibres with resting potentials between -55 and -60 mV.

The absence of action potentials in tonic fibres of rat extraocular muscles contrasts with the report that action potentials are present in multiply innervated fibres of cat extraocular muscles (Bach-y-Rita & Ito, 1966). This disagreement may be related to a difference in the type of multiply innervated fibres analysed in both studies either because a different species was examined or because different regions of the muscle containing different fibre types were explored (Peachey, 1971; Chiarandini & Davidowitz, 1979). The possibility that the fibres studied by Bach-y-Rita and Ito (1966) were wrongly identified as multiply innervated should be also considered (Pilar, 1967; Barmack, Bell & Rence, 1971).

Some of the electrical properties of tonic fibres of rat extraocular muscles such as high R_{eff} and graded responses with relatively slow time courses are characteristic of cells in which Ca channels play a role in electrogenesis (Hille, 1978). However, the apparent lack of sensitivity of the slow peak potentials to D-600 eliminates Ca as the primary carrier of the inward current during this phenomenon.

On the other hand the disappearance of the slow peak potentials in Na-free saline and their blockade by TTX implicates Na as the primary carrier of the inward current. The small, rounded responses observed in high concentrations of TTX when the fibres were stimulated with large depolarizing pulses were eliminated by combined application of TEA and TTX, which suggests that they involve the K-delayed rectifier rather than a TTX-resistant Na conductance or a small Ca conductance.

The concentration of TEA used in this study was relatively low (25 mM) but in frog twitch fibres it is enough to reduce delayed rectification by about 80% (Stanfield, 1970). TEA produced an increase of about five times in the duration of the

slow peak potential which indicates that the delayed rectifier is responsible in part for the falling phase of the response. Unexpectedly, it was observed that in fibres exposed to Na-free saline delayed rectification was less evident and larger depolarizations were needed to initiate it. Whether this results from a change in threshold or amplitude of the delayed rectification or both, is unknown. Related to this observation is the report that in heart muscle Na removal reduces the amplitude of i_{K_2}, a slow K current with outward-rectifying properties (Noble & Tsien, 1969).

Although tonic fibres have voltage dependent Na and K channels which have pharmacological properties similar to the respective channels of amphibian and mammalian twitch fibres (Stanfield, 1970; Adrian & Marshall, 1977), tonic fibres are unable to produce action potentials. A very low density of Na channels could be an explanation for the absence of a regenerative response. Direct measurements of R_{eff} in control and Cl-free saline have confirmed the previous suggestion (Chiarandini & Stefani, 1979) that tonic fibres of extraocular muscles have an undetectable permeability to Cl ions. This apparent lack of permeability to Cl, and the absence of action potentials in addition to the previously described capability of producing maintained tensions (Chiarandini, 1976) indicate the close relationship between tonic fibres of amphibian muscles and mammalian extraocular muscles.

The authors wish to thank Dr J. Wiggins for helpful discussions and Miss K. Rhee for help in the preparation of the manuscript. This study was supported by U.S.P.H.S. grants EY 01297 and EY 07009 and, partially, by an unrestricted grant from Research to Prevent Blindness, Inc.

REFERENCES

ADRIAN, R. H. & MARSHALL, M. W. (1977). Sodium currents in mammalian muscle. *J. Physiol.* **268**, 223–250.

BACH-Y-RITA, P. (1971). Neurophysiology of eye movements. In *The Control of Eye Movements*, ed. BACH-Y-RITA, P., COLLINS, C. C. & HYDE, J. E., pp. 7–45. New York and London: Academic.

BACH-Y-RITA, P. ITO, F. (1966). In vivo studies on fast and slow muscle fibers in cat extraocular muscles. *J. gen. Physiol.* **49**, 1177–1198.

BARMACK, N. H., BELL, C. C. & RENCE, B. G. (1971). Tension and rate of tension development during isometric responses of extraocular muscle. *J. Neurophysiol.* **34**, 1072–1079.

BONDI, A. Y. & CHIARANDINI, D. J. (1978). Electrophysiological studies of rat extraocular muscles. *Physiologist, Wash.* **21**, 11.

CHIARANDINI, D. J. (1976). Activation of two types of fibres in rat extraocular muscles. *J. Physiol.* **259**, 199–212.

CHIARANDINI, D. J. & DAVIDOWITZ, J. (1979). Structure and function of extraocular muscle fibres. In *Current Topics in Eye Research*, vol. 1, ed. ZADUNAISKY, J. A. & DAVSON, H., pp. 91–142. New York: Academic.

CHIARANDINI, D. J. & STEFANI, E. (1979). Electrophysiological identification of two types of fibres in rat extraocular muscles. *J. Physiol.* **290**, 453–465.

DÖRRSCHEIDT-KÄFER, M. (1977). The action of D600 on frog skeletal muscle: facilitation of excitation-contraction coupling. *Pflügers Arch.* **369**, 259–267.

FERNAND, V. S. V. & HESS, A. (1969). The occurrence, structure and innervation of slow and twitch muscle fibres in the tensor tympani and stapedius of the cat. *J. Physiol.* **200**, 547–554.

FLOYD, K. (1973). Cholinesterase activity in sheep oesophageal muscle. *J. Anat.* **116**, 357–373.

HESS, A. & PILAR, G. (1963). Slow fibres in the extraocular muscles of the cat. *J. Physiol.* **169**, 780–798.

HILLE, B. (1978). Ionic basis of resting and action potentials. In *Handbook of Physiology. The Nervous System 1*, vol. 1 (revised), ed. BROOKHART, J. M. & MOUNTCASTLE, V. B., pp. 99–136. Bethesda, Md: American Physiological Society.

KENYON, J. L. & GIBBONS, W. R. (1977). Effects of low-chloride solutions on action potentials of sheep cardiac Purkinje fibres. *J. gen. Physiol.* **70**, 635–660.

MATYUSHKIN, D. P. (1964). Motor systems in the oculomotor apparatus of higher animals. *Fedn Proc.* **23**, T1103–1106.

MAYR, R. (1971). Structure and distribution of fibre types in the external eye muscles of the rat. *Tissue & Cell* **3**, 433–462.

NOBLE, D. & TSIEN, R. W. (1969). Outward membrane currents activated in the plateau range of potentials in cardiac Purkinje fibres. *J. Physiol.* **200**, 205–231.

PEACHEY, L. (1971). The structure of the extraocular muscle fibers of mammals. In *The Control of Eye Movements*, ed. BACH-Y-RITA, P., COLLINS, C. C. & HYDE, J. E., pp. 47–66. New York and London: Academic.

PILAR, G. (1967). Further study of the electrical and mechanical responses of slow fibers in cat extraocular muscles. *J. gen. Physiol.* **50**, 2289–2300.

STANFIELD, P. R. (1970). The effect of the tetraethylammonium ion on the delayed currents of frog skeletal muscle. *J. Physiol.* **209**, 209–229.