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THE MAINTENANCE OF RESTING POTENTIALS IN GLYCEROL-TREATED MUSCLE FIBRES

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SUMMARY

1. A modification of a previously published method for the disruption of the T-tubules of frog skeletal muscle is described. The modification permits the disruption of the T-tubules without the decline in resting potentials which was reported previously.

2. The method for the disruption of the T-tubules involves the washout of glycerol following loading in a 400 mM glycerol Ringer solution. The modification consists of elevating the concentration of divalent cations in the Ringer used for glycerol washout.

3. The optimum concentrations are 5 mM-Ca^{2+} and 5 mM-Mg^{2+} added as their chloride salts. Neither 10 mM-Ca^{2+} nor 10 mM-Mg^{2+} are as effective as the combination of each at 5 mM. Other concentrations gave less satisfactory results.

4. The use of the modified technique provides a preparation which maintains 85–90 mV resting potentials for up to 6 or 8 hr but which will not contract in response to membrane depolarization.

INTRODUCTION

The glycerol-treated muscle preparation (Howell & Jenden, 1967; Krolenko, Adamjan & Shvinka, 1967; Krolenko, 1968; Howell, 1969) has been widely used to study the properties of the transverse tubular system and the surface membrane of skeletal muscle (Brautaset & Nicolysen, 1968; Nakajima, Nakajima & Peachey, 1969; Stefani & Steinbach, 1968; Eisenberg & Gage, 1969; Gage & Eisenberg, 1969*a*, *b*; Ildefonse, Pager & Rougier, 1969; Van der Kloot, 1969; Bryant, 1970; Henderson, 1970;

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Yamada, 1970). However, the low values and large variation of resting potentials in the treated fibres (Howell, 1969; Eisenberg & Gage, 1969) have limited the effective application of the technique to experiments in which the properties of individual fibres could be studied. The experiments reported here were undertaken in order to find conditions under which the T-system could be disrupted by glycerol washout without affecting the resting potentials. A preliminary report of this work has appeared (Howell, Vaughan & Eisenberg, 1970).

METHODS

Except where specifically indicated otherwise, all experiments were performed on bundles of four to forty fibres dissected from the dorsal head of semitendinosus muscle of *Rana pipiens*. Tension measurements were made with an RCA 5734 transducer. Resting potentials were measured with glass micropipettes filled with 2·5 M-KCl and having resistances of 8–20 MΩ. The input stage was the same as that used by Eisenberg & Gage (1969). A continuous flow system was used in the muscle bath which enabled us to change solutions in 30 sec. Stimulation was provided through platinum wires mounted in the bottom of the bath. A few experiments were done using the surface fibres of the sartorius; in these experiments no tension measurements were made.

The basic Ringer solution contained 115 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$, D-tubocurarine chloride, 10^{-5} g/ml., and 3 mM phosphate added as NaH_2PO_4 and Na_2HPO_4 to give a pH of $7·2 \pm 0·05$. The glycerol Ringer had the same constituents, plus 400 mM glycerol. In Ringer modified by addition of excess $CaCl_2$ or of $MgCl_2$ Tris buffer was used (also at pH 7·2). The solution compositions are given in detail in Table 1.

RESULTS

Following dissection, the bundles were transferred to the experimental chamber without being passed through an air/water interface. The lengths of the muscles were adjusted to produce maximum twitch tension and the stimulus intensity was made just supramaximal. Twitch and tetanus tensions were measured and resting potentials were sampled. The solution flowing into the chamber was then changed from normal Ringer to a Ringer solution which contained 400 mM glycerol in addition to the constituents of the normal Ringer. The muscles were exposed to this solution for at least 50 min before being returned to glycerol-free Ringer, hereafter called the washout solution. Washout solutions were modified by the addition of varying amounts of $CaCl_2$ and $MgCl_2$ to bring the divalent cation concentrations to the values indicated in Table 2. Resting potentials were sampled immediately after the twitch declined in amplitude to less than 1% of its pre-treatment value and at subsequent intervals indicated in Table 2. The decline in resting potentials which occurred when the glycerol washout solution was normal Ringer is indicated in the first row of Table 2

TABLE 1. Solution composition in m-mole/l.

Solution code	Description	Na^+	K^+	Ca^{2+}	Mg^{2+}	Sucrose	Cl^-	$H_2PO_4^-$	HPO_4^{2-}	$TrisH^+$	Tris
A	Ringer	119·5	2·5	1·8	—	—	121·1	1·52	1·48	—	—
B	3 mM-Ca Ringer	115	2·5	3	—	—	126·2	—	—	2·65	0·35
C	5 mM-Ca Ringer	115	2·5	5	—	—	130·2	—	—	2·65	0·35
D	10 mM-Ca Ringer	115	2·5	10	—	—	140·2	—	—	2·65	0·35
E	10 mM-Mg Ringer	115	2·5	1·8	10	—	140·2	—	—	2·65	0·35
F	3 mM-Ca/5 mM-Mg Ringer	115	2·5	3	5	—	136·2	—	—	2·65	0·35
G	5 mM-Ca/5 mM-Mg Ringer	115	2·5	5	5	—	140·2	—	—	2·65	0·35
H	Isotonic 5 mM-Ca/5 mM-Mg Ringer	102·5	2·5	5	5	—	127·7	—	—	2·65	0·35
I	Hypertonic sucrose Ringer	115	2·5	1·8	—	24·6	123·8	—	—	2·65	0·35
J	Hypertonic NaCl Ringer	127·3	2·5	1·8	—	—	136·1	—	—	2·65	0·35

and is similar to previously reported values (Howell, 1969; Eisenberg & Gage, 1969). The addition of as little as 1·2 mM-Ca^{2+} (solution B) to the washout solution produced a noticeable improvement in resting potentials. Additions of 3·2 and 8·2 mM-Ca^{2+} (solutions C and D) seemed to produce some further improvement but the resting potentials still declined with time. Addition of 10 mM-Mg^{2+} (solution E) also seemed to help, particularly in improving the stability of resting potentials over a period of hours. We

TABLE 2. Resting potentials after glycerol washout in Ringer solutions containing varying concentrations of divalent cations

	Before	Immediately after	1 hr later	2 hr later	3–4 hr later	6–8 hr later	15 hr later
Normal Ringer	90 + 0·9 (21)	74 ± 1·1 (29)	—	71 ± 2·3 (13)	—	—	—
3 mM-Ca	89 + 0·4 (39)	80 ± 0·9 (39)	59 ± 1·6 (22)	75 ± 3·2 (8)	—	—	—
5 mM-Ca	90 ± 0·4 (18)	87 ± 0·8 (19)	77 ± 1·3 (8)	—	—	—	—
10 mM-Ca	90 ± 0·6 (18)	84 ± 1·2 (25)	78 ± 1·7 (11)	—	—	—	—
10 mM-Mg	92 ± 0·5 (9)	81 ± 0·8 (10)	—	81 ± 2·6 (11)	72 ± 3·3 (4)	87 ± 1·9 (7)	—
3 mM-Ca + 5 mM-Mg	89 ± 0·6 (26)	80 ± 0·8 (41)	85 ± 1·5 (23)	83 ± 2·7 (21)	79 ± 2·9 (8)	75 ± 1·1 (14)	68 ± 5·1 (16)
5 mM-Ca + 5 mM-Mg	89 ± 0·6 (33)	88 ± 0·8 (23)	87 ± 1·3 (27)	—	—	91 ± 1·4 (10)	—

Resting potentials in mV ± S.E. of mean. Number of fibres sampled indicated in parentheses.

found that there was no significant decrement in resting potentials when the glycerol washout was done in a Ringer solution containing 5 mM Ca^{2+} and 5 mM-Mg^{2+} (Table 1, solution G). If the washout solution contained an elevated concentration of divalent cations, the twitch disappeared more slowly – in about 15 min instead of 7. The muscles still became opaque and granular in appearance under the dissecting microscope and exhibited the same physiological properties as do muscles subjected to glycerol washout in normal Ringer. We observed a fall in capacitance to about 2·3 $\mu F/cm^2$ using 3 mM-Ca^{2+} and 5 mM-Mg^{2+} in the glycerol washout solutions (Vaughan, Howell & Eisenberg, 1970). There was a tendency for the twitch response to recover over a period of hours, but this recovery generally amounted to less than 5% of the pretreatment tension. The maximum recovery observed was to 10% of the original value but this was observed only once.

In the experiments of Table 2 the muscles were continuously exposed to the modified Ringer after glycerol washout. Experiments done on sartorius muscles (Table 3) indicate that resting potentials remain high in muscles returned to normal Ringer solution, if the glycerol washout is done in the presence of elevated divalent cation concentration. In these experiments the muscles were returned to normal Ringer after 1 hr in the high Ca^{2+}/Mg^{2+} Ringer. Resting potentials were sampled just before

TABLE 3. Resting potentials in normal Ringer after glycerol washout in modified Ringer

Ringer before treatment	1 hr after transfer from glycerol Ringer to solution G	1 hr later after being soaked for 1 hr in normal Ringer
94·4 ± 0·5 (22)	86·6 ± 0·6 (20)	90·4 ± 0·6 (39)

TABLE 4. Resting potentials after glycerol washout in Ringer made slightly hypertonic with sucrose

Ringer before treatment	1 hr after transfer from glycerol Ringer to solution I	2 hr later in solution I
93·6 ± 0·5 (25)	64·9 ± 1·9 (44)	56·6 ± 2·4 (55)

muscles were transferred from the high Ca^{2+}/Mg^{2+} Ringer into the normal Ringer instead of immediately after the disappearance of the twitch as was done in the experiments on bundles. A few similar experiments were carried out with bundles to confirm these results obtained on the sartorius.

In order to exclude the unlikely possibility that the improvement in the resting potentials might be due to the increase in osmolarity produced by the addition of the calcium and magnesium salts, several sartorius muscles were put in washout solutions modified by the addition of sucrose instead of calcium and magnesium chloride (solution I). The amount of sucrose added, 24·6 mM, was calculated to be equal in osmolarity (ignoring osmotic coefficients) to the added calcium and magnesium chlorides which gave optimum protection of resting potentials. The results appear in Table 4 and indicate that the increased osmolarity provided by the sucrose did not prevent the resting potentials from falling as a consequence of glycerol washout. Similar experiments on fibre bundles with Ringer solutions made hypertonic to the same extent by the addition of extra NaCl instead of sucrose (solution J), also indicated that the increase in osmolarity itself

could not prevent the decline in resting potentials during glycerol washout. However, when the Ringer used for glycerol washout contained 5 mM-Ca and 5 mM-Mg but with the NaCl concentration reduced to make the solution isosmotic with normal Ringer (solution H), an interesting phenomenon was observed. If these fibres were sampled for resting potentials immediately after the twitch disappeared, a puckering of the cell surface invariably appeared at the site of the electrode penetration. This puckering often developed into a retraction clot. The resting potentials of such fibres declined rather rapidly so that when fibres of the bundle were sampled an hour or two later, resting potentials fell into two distinct categories (above 80 mV and below 65 mV) which corresponded to the fibres which had previously been penetrated with the micro-electrode and to those which had not. These results suggest that there is a period of time early in the glycerol washout, namely immediately after the disappearance of the twitch, when the fibres are very susceptible to mechanical injury and that this susceptibility is reduced by the increased osmolarity associated with the addition of 3·2 mM-$CaCl_2$ and 5 mM-$MgCl_2$ to the washout solution.

DISCUSSION

The results reported here suggest that the glycerol treatment can be improved by increasing the divalent cation concentration in the Ringer solution used during the glycerol washout. The resulting preparation has quite normal resting potentials, but the link between excitation and contraction has been broken.

The use of the glycerol treatment to measure properties of whole muscles requires the treatment to have a uniform effect on all of the fibres in the preparation. Our previous experience with sartorius muscles had indicated that the disruption of the T-tubules is sometimes incomplete, the deeper fibres of the muscle tending to have more intact T-system remaining than the surface fibres (Eisenberg & Eisenberg, 1968; Howell, 1969). Consequently we used either surface fibres from the deep side of the sartorius or small bundles of fibres, the size of toe muscles or smaller, dissected from semitendinosus muscles. In the bundles the disappearance of the twitch was completely dependable. We would like to emphasize that if the glycerol treatment is applied to larger muscles or muscles of different morphology, it is necessary for the investigator to demonstrate that the tubular system is disrupted throughout the preparation.

The tendency of the muscle fibres to be mechanically fragile during the early stages of glycerol washout, a phenomenon which we saw particularly clearly under certain conditions described above, is not surprising. Following the loading of the fibres with 400 mM glycerol, the Ringer solution in

which the glycerol washout is done appears quite hypotonic to the fibres and they undergo an initial swelling which disappears as the glycerol washes out. This has been clearly described in single fibres by Krolenko & Adamjan (1967). It seems likely that this swelling is responsible for the fragility of treated fibres. Because of this transient fragility it seems wise to postpone any manipulation of the muscles until 1 hr after the glycerol washout has begun.

The long term maintenance of resting potentials following glycerol washout in the high Ca^{2+}/Mg^{2+} Ringer is good. We observed 90 mV resting potentials for up to 6–8 hr after tubular disruption. Resting potentials tended to rise rather than to fall in the period of time following the disappearance of the twitch. We observed this tendency especially in solutions containing magnesium (Table 2).

There have been observations made on different preparations which suggest that there may be slow morphological and physiological changes which take place over a period of hours following T-tubule disruption (Krolenko, 1969; Henderson, 1970). The slight tendencies of the resting potentials to rise and of the twitch to reappear in our experiments may reflect these changes. However, considering the differences in species, muscles, and glycerol concentrations used, it is difficult to make direct comparisons with other reported observations. The slow changes may prove interesting but they cannot be interpreted at this time. In any case, we have never observed a substantial restoration of excitation–contraction coupling in our experiments, which suggests that the T-tubules have very little tendency to restore their connexions with the extracellular space.

The specific contribution of calcium and magnesium to the protection of the resting potentials in this modified procedure is certainly not clear. The fact that an equimolar mixture of calcium and magnesium is more effective than either alone at the same total concentration does seem to indicate that each cation is playing an individual role. If anything at all can be inferred from the data in this regard, it is that calcium is required for protection against the initial decline in resting potentials which occurs during the disruption of the T-system, whereas magnesium contributes to the maintenance of resting potentials following disruption. However, we have not excluded the possibility that the contribution of the magnesium arises from osmotic, ionic strength or other effects. Despite the fact that the mechanisms are not clear either for the protective effect of the divalent cations or even for the T-tubular disruption itself, the technique is likely to be of continuing value in the study of the properties of the T-system, the surface membrane, and of the processes of excitation–contraction coupling.

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