COMPARISON OF GLYCEROL TREATMENT IN FROG SKELETAL MUSCLE AND MAMMALIAN HEART
An Electrophysiological and Morphological Study

G. NIEMEYER and W. G. FORSSMANN

From the Institute of Physiology, University of Berne, and the Institute of Histology, Medical School, University of Geneva, Switzerland. Dr. Forssmann's present address is the Department of Anatomy, University of Heidelberg, Heidelberg, Germany. Dr. Niemeyer's present address is the National Eye Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT
Frog skeletal muscle and mammalian heart muscle were studied in vitro before and after glycerol treatment. Loss of contractility, changes in the action potential and disruption of the T system were observed in skeletal muscle cells. In mammalian heart muscle the T system was not disrupted with hypertonic glycerol treatment, and no significant electrophysiological changes were observed. The continuity between the T system and the extracellular space was investigated by diffusion tracer methods. Decrease of contractility during the hypertonic phase in the glycerol treatment was found to depend on tonicity. The results of this study clearly show that not only are there differences in morphology between skeletal and cardiac muscle, but there are also differences in the resistance to osmotic changes.

INTRODUCTION
Since discussion about the functional and morphological differences between skeletal and heart muscle is still open, it seemed to be of interest to compare the behavior of both tissues after exposure to hypertonic glycerol solutions. It is well known that the transverse tubular system (T system) as a part of the sarcoplasmic reticulum (the term sarcoplasmic reticulum is used according to Porter and Palade [32], see also Forssmann and Girardier [9]) is an essential part of the excitation-contraction coupling mechanism in skeletal muscle (20). It can be damaged in frog skeletal muscle by means of a sudden change from hypertonic glycerol-Ringer's to normal Ringer's (19). This procedure is called "glycerol treatment." This method has been applied to mammalian heart muscle, and in the present study contraction was observed and transmembrane potentials were measured before and after glycerol treatment. The structural status of the T system was followed throughout the whole experiment by a diffusion tracer method. To indicate whether the connection between the T system and the extracellular space was intact, a soaking-out procedure for the tracer substance was used.

MATERIAL AND METHODS
Electrophysiology
Muscle trabeculae (average diameter 0.7 mm, average length 8 mm) from the right ventricle of sheep and calf hearts were cut out within 40 min after slaughtering. All experiments were done within 7 hr after the preparation. Equilibration in the different solutions at 23° or 35°C was performed in a perfused Perspex chamber, in which stimulating
and recording electrodes were placed (26). The muscle strips were driven at a frequency of 0.5–1 cycle/sec by extracellular electrodes from a Grass stimulator (Grass Instrument Co., Quincy, Mass.), through a stimulus isolation unit. The transmembrane potentials were recorded differentially with glass microelectrodes filled with 3 M KCl, by means of a cathode follower and a Tektronix 502 oscilloscope (Tektronix, Inc., Beaverton, Ore.). The electrode resistance was between 12 and 30 MΩ. The contraction was continuously observed with a binocular microscope (magnification 40). After equilibration in normal Tyrode's solution (solution I) for at least 30 min, the perfusion solution was abruptly changed either to 400 mM glycerol–Tyrode's or to 1000 mM glycerol–Tyrode's solution (solution II or III). The preparation was perfused with the glycerol-containing solution for 60 min and then the perfusion was abruptly changed back to normal Tyrode's solution. In order to hyperpolarize the preparation of heart muscle the sucrose gap method of Wood et al. (36) was employed. In this technique a three-compartment chamber was used, the middle chamber having a width of 2 mm. This middle chamber was perfused with isotonic sucrose solution, providing a high extracellular resistance. The other two chambers were perfused with Tyrode's solution. The length of the fiber extending into the front chamber was 1 mm. Constant current pulses were applied through a 50–100 kΩ series resistor to the posterior chamber, and the changes in the membrane potential were measured in the anterior chamber. When this method was used, it was also possible to apply glycerol treatment. In order to do this, all three chambers were perfused with the appropriate glycerol solution. In the sucrose gap technique, hyperpolarizations of less than 10 mV were used in order to have fairly uniform voltage distribution along the fiber (calculation from cable equations, McGuigan, unpublished). In all experiments the electrode position remained unchanged in the middle of the front segment of the fiber. To calculate the time constant, a value of 69% of the final value was taken. This was calculated by Weidmann by means of the superimposition method from Hodgkin and Rushton, Table I (17).

To check the behavior of frog twitch muscle, bundles containing 5–30 fibers were prepared from m. semitendinosus of Rana temporaria. Perfusion with Ringer's solution (V) and glycerol solution (VI) was performed at room temperature in the same way as in heart muscle.

Solutions

I. Tyrode's, bubbled with 95% O₂/5% CO₂, containing (in millimoles per liter): NaCl 136.9, KCl 5.4, NaHCO₃ 11.9, Na₂HPO₄ 0.4, CaCl₂ 1.8, MgCl₂ 0.5, and glucose 1 g.

II. The same as I, with the addition of 400 millimoles glycerol/liter.

III. The same as I, with the addition of 1000 millimoles glycerol/liter.

IV. Sucrose with ions, oxygenated as I, containing (in millimoles per liter): KCl 2.1, CaCl₂ 1.1, glucose 1 g, sucrose 94.5 g/liter.

V. Ringer's, containing (in millimoles per liter): NaCl 115.5, KCl 2.5, CaCl₂ 1.8, NaH₂PO₄ 0.9, Na₂HPO₄ 2.2.

VI. The same as V, containing 400 millimoles glycerol/liter.

Electrophysiology

The solutions used for the physiological experiments were also used during the preparation for electron microscopy, and in some cases the same piece of tissue was utilized for both studies. At different stages during the experiment (see Table I) the physiological solutions were replaced by a 2% glutaraldehyde phosphate–buffered fixative (34). After fixation for 1 hr the tissue was cut into small blocks which were then washed three times in phosphate buffer solution. The blocks were then postfixed with phosphate-buffered osmium tetroxide (29). If a tracer experiment had been done, the blocks were first treated for the histochemical demonstration of horseradish peroxidase (see below). After these procedures the blocks were dehydrated and embedded in Epon (28), sectioned on a LKB ultramicrotome (LKB Produkter, Stockholm, Sweden), and contrasted after the method of Karnovsky (23). The sections were examined with a Zeiss EM 9 or a Philips EM 300 electron microscope. Semithin sections were observed and photographed in a Zeiss photomicroscope with a phase-contrast device.

The diffusion tracer (horseradish peroxidase, HRP) technique of Karnovsky (24) and Graham and Karnovsky (15) was applied with some slight modifications (Forsmann [8]). The histochemical controls were performed as indicated by Karnovsky (25). In all experiments the quantity of HRP added to the solutions was between 2 and 5 mg HRP/ml.

RESULTS

Electrophysiology

FROG SKELETAL MUSCLE: The results as obtained by others (3, 5, 11, 18, 19) could be fully confirmed. After glycerol treatment (using solutions V and VI), the mechanical response to electrical stimulation disappeared completely within 40 min. Single fibers twitched spontaneously during the first minutes after the removal of the hypertonic glycerol-Ringer's. The action potential

G. NIEMEYER AND W. G. FORSSMANN Glycerol Treatment in Muscle and Heart 229
TABLE I

_Frog Skeletal Muscle and Mammalian Heart Muscle: Electrophysiological Results with Glycerol Treatment_

**Frog Skeletal Muscle**

<table>
<thead>
<tr>
<th></th>
<th>Ringer's solution (V)</th>
<th>400 mM glycerol-Ringer's (VI)</th>
<th>After glycerol (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractility</td>
<td>normal</td>
<td>normal</td>
<td>absent</td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>normal</td>
<td>normal</td>
<td>normal, reduced</td>
</tr>
<tr>
<td>Action potential</td>
<td>normal</td>
<td>normal</td>
<td>present with highly decreased early afterpotential</td>
</tr>
<tr>
<td>Late afterpotential</td>
<td>normal</td>
<td>normal</td>
<td>absent</td>
</tr>
</tbody>
</table>

**Mammalian Heart Muscle**

<table>
<thead>
<tr>
<th></th>
<th>Tyrode's solution (I)</th>
<th>400 mM glycerol-Tyrode's (II)</th>
<th>Tyrode's (I) after glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractility</td>
<td>normal</td>
<td>slightly reduced</td>
<td>normal</td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Action potential</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Upstroke velocity</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Time constant of hyperpolarization</td>
<td>4.9 ± 0.99 msec</td>
<td>---</td>
<td>5.2 ± 0.4 m sec</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Tyrode's solution (I)</th>
<th>1000 mM glycerol-Tyrode's (III)</th>
<th>Tyrode's (I) after glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractility</td>
<td>normal</td>
<td>greatly reduced, sometimes initially absent</td>
<td>normal</td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Action potential</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Upstroke velocity</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>

was still present, but the early afterpotential was markedly decreased and the late afterpotential, following a tetanic stimulation (50–100 cycle/sec), disappeared completely. After this change (see Fig. 1, Table I), the resting membrane potential was in general slightly reduced, occasionally to a value above the mechanical threshold. These changes were irreversible.

**Mammalian Heart Muscle:** Similar results were obtained in 17 experiments with sheep or calf trabeculae. In the hypertonic glycerol solutions, a marked decrease of contraction (see Table I) and a decrease in fiber diameter of the order of 10% was observed. Both effects were more pronounced in the solution with the higher tonicity (solution III). With solution III, the contractility sometimes disappeared completely for several minutes in the hypertonic phase of the
treatment, but always recovered in normal Tyrode's.

The resting membrane potential showed minor variation after every change of solution. Since, in several experiments, a microelectrode remained in the same cell for as long as 90 min, it was possible to follow these changes in detail. The action potentials did not show any remarkable change in shape, amplitude, upstroke velocity or duration during and after application of either 400 or 1000 mm glycerol solution. The present method would have allowed detection of changes of +7 mv in overshoot, +10% in upstroke velocity, and ±50 msec in duration. No further changes were observed when the time of observation, after glycerol removal, was increased to 4 hr. Even when glycerol perfusion time was increased from 60 min to 4 hr, the results were similar. A reduction in temperature to 23°C did not influence these results.

Hyperpolarizing current pulses produced a transmembrane voltage displacement of a similar amplitude before and after glycerol treatment (see Fig. 2, C and D), the time constant was 4.9 ± 0.99
FIGURE 3 Frog skeletal muscle after incubation for 30 min in 400 mM glycerol-Ringer’s and 30 min in normal Ringer’s. Fig. 3a shows a localized swelling of a T tubule (arrow); Fig. 3b shows disrupted T tubules (T) showing no clear membrane limit with the L system. X 30,000.

FIGURE 4 Frog skeletal muscle, incubated for 30 min in Ringer’s, followed by 30 min in 400 mM glycerol-Ringer’s containing 3 mg/ml horseradish peroxidase, and then fixed. Note the normal structure of the T system and the tracer substance filling all T tubules. X 30,000.
FIGURE 5  Frog skeletal muscle incubated for 30 min in Ringer’s, 30 min in 400 mM glycerol-Ringer’s, 30 min in Ringer’s, and finally 30 min in Ringer’s containing peroxidase. T tubules are not filled with tracer substance. Tracer is found only in the interstitial space. × 32,000.

FIGURE 6  Frog skeletal muscle incubated throughout the glycerol treatment with solutions containing the tracer, then washed for 30 min in normal Ringer’s. The tracer is washed out from the interstitial space but not from the T system. × 32,000.
FIGURE 7  Heart muscle incubated for 60 min in normal Tyrode's solution. Note the normal T system (T) and the normal chromatin in the cell nucleus (N). × 24,000.

FIGURE 8  Heart muscle incubated for 40 min in 400 mM glycerol-Tyrode's solution. Note the swelling of the T system (T) and the condensed configuration of chromatin in cell nucleus (N) as a sign of hyper-tonicity. × 24,000.
**Figure 9** Heart muscle incubated for 30 min in Tyrode's solution, and for 30 min in 400 mM glycerol-Tyrode's with peroxidase. All T tubules are labeled by the tracer substance, and there is no marked dilatation of T tubules (T). × 18,000.

**Figure 10** Heart muscle incubated in Tyrode's with peroxidase after glycerol treatment with 1000 mM glycerol-Tyrode solution. A slight swelling of T tubules (T) persists, but no disruption is observed; all T tubules are labeled. × 22,000.
msec before and 5.2 ± 0.49 msec after (mean values and sd, one experiment, 10 impalements; the difference is not statistically significant).

**Morphology**

**Frog Skeletal Muscle:** Examination of frog skeletal muscle in the different glycerol experiments showed that the results of Howell and Jenden (19) could be reproduced (Figs. 3a and 3b). As is seen in Table II and Fig. 4, there are no ultrastructural alterations in isolated fibers during the whole period of incubation in either Ringer's solution or Ringer's solution containing 400 mm glycerol and HRP, even if the muscles were fixed some hours after incubation. The maximal incubation time in disruption experiments was 3 hr.

If the HRP was added to the solutions as a tracer, it penetrated both the interstitial space and the T system even if the muscle fibers were already incubated in glycerol-Ringer's (Fig. 4). In all of these stages the tracer could be washed out by using the same solution without HRP, demonstrating that the T system was intact throughout the incubation with Ringer's or glycerol-Ringer's.

If the muscle fibers were prepared for electron-microscopy 30 min after the removal of glycerol, the T tubules were locally swollen or disrupted, forming a large clear space from the Z band sometimes reaching as far as the next sarcomere (Fig. 3b). Frequently the T membrane was observed to be disrupted, and continuity between T and L cisternae appeared to result.

The disruption of the T system was demonstrated with tracer experiments. It was observed that the tracer did not penetrate into the T tubules after glycerol treatment (Fig. 5), and that the tracer substances when present in glycerol-Ringer's, as well as in normal Ringer's during the period of disruption, could not subsequently be washed out from the T tubules (Fig. 6). However, the tracer was washed out completely from the interstitial space after several changes of the Ringer's without HRP (Fig. 6).

**Sheep and Calf Ventricular Fibers:** The investigation of mammalian heart muscle showed that the T system was not disrupted if the same treatment, or even treatment with 1000 mm glycerol, was carried out. In the controls the heart muscles did not change in their morphological features during several hours of incubation in normal, oxygenated Tyrode's at 35°C (Fig. 7). If glycerol was added, a swelling of the T system was observed (Fig. 8).

Horseradish peroxidase could be added at any stage of the glycerol treatment or in control incubations in normal Tyrode's (Figs. 9 and 10), and it was found to diffuse freely to all of the T tubules. If the fibers were washed with tracer-free Tyrode's before fixation, a complete removal of HRP was possible in all stages of the experiment (Table II). This indicates that T tubules are only slightly swollen after glycerol treatment, and disruption never occurred in our experiments.

**Table II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Skeletal muscle</th>
<th>Heart muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tracer in T system</td>
<td>Tracer in interstitium</td>
</tr>
<tr>
<td>Control electrolyte solution</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Electrolyte solution containing HRP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol electrolyte solution containing HRP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Electrolyte containing HRP after glycerol treatment*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol treatment with solutions all containing HRP, then washing in HRP-free electrolyte solution</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* For explanation of glycerol treatment, see Introduction.
DISCUSSION

In frog skeletal muscle a change from electrolyte solution to a hypertonic, glycerol-containing electrolyte solution does not produce physiological or morphological alterations. However, if the preparation is brought back to normal Ringer's solution, drastic changes in both physiological and morphological characteristics are observed.

In 1967, Howell and Jenden (19) first demonstrated electron microscopically that the failure of excitation-contraction coupling was associated with a disruption of the T system. Mechanical response of glycerol-treated fibers could only be obtained with caffeine. In the same year, Eisenberg and Gage (5, 11) examined the electrical properties of frog muscle after disruption of the T system and were able (6) to differentiate between the surface and tubular fraction of the membrane capacity and specific ionic conductances for K⁺ and Cl⁻. For further details concerning physiological and morphological properties of the T system, see references 3, 4, 6, 12, 13, 18 and 31.

On the other hand, Howell (18) has shown that T-tubular disruption is observed only with an abrupt, but not with a stepwise, removal of glycerol-Ringer's. The same effect could also be obtained with urea, but not with sucrose or with fast-penetrating substances such as ethylene glycol. He also investigated slow muscle fibers of the frog and found that they are not sensitive to glycerol treatment.

Our own results on frog skeletal muscle are in agreement with those discussed above. The morphological investigations carried out on frog skeletal muscle were especially related to the problem of finding when the disruption of the tubular system occurs. It was previously shown by Eisenberg and Eisenberg (3, 4) using peroxidase and by Nakajima et al. (31) using ferritin as diffusion tracers, that the T-tubule compartment is separated from the interstitial space by glycerol treatment. Our present study reveals beyond any doubt that the lumen of the T tubules must become discontinuous during the removal of glycerol, since we have demonstrated that peroxidase can not only penetrate to the T system before the disruption but can also be removed by soaking in HRP-free solution. If the T system is disrupted, peroxidase is removed only from the interstitial space, but never from the T system. This clearly demonstrated that the diffusion of tracers is abolished in both directions.

The function of the T system in skeletal muscle with respect to excitation-contraction coupling is quite well defined by the work of Huxley et al. (20-22) and the more recent work dealing with glycerol treatment. However, little is known about the role of the T system in mammalian heart (7, 9), even though its structure has been well studied. In contrast to skeletal muscle, heart muscle cells are smaller and the action potential is so much longer that an implication of a T system in excitation-contraction coupling might not be required. Thus, the function of the T system in mammalian heart still remains obscure. In addition to the recently reported findings concerning the response to local stimulation in sheep heart by Mueller (30) and the detailed morphological description of the T system in rat heart (for literature, see Fawcett and McNutt [7]), the results presented here again emphasize the different characteristics of this intracellular membrane system in heart. Since the experiments carried out in 400 mM glycerol solution were not effective in heart tissue, we increased the tonicity by adding 1000 mmols glycerol/liter Tyrode's. Even with this increase of tonicity, the only morphological change caused by glycerol treatment was a slight swelling of the T system. Swelling of the T system could be observed as an effect of osmotic or ionic changes (1, 27), and increase of osmolarity of the bathing solution also produces swelling of the T system in skeletal muscles (2, 10).

During perfusion with 400 mM glycerol solution and for a short time after the change back to normal Tyrode's, a decrease in contraction was observed. In the presence of 1000 mM glycerol-Tyrode's, the contractions sometimes disappeared for periods of 5-15 min.

Since this decrease in contraction may not be related to the T system, other possibilities have to be considered. A loss of contraction may occur as a simple consequence of removal of cell water, explained by changes in filament lattice spacing. In fact, a decrease in filament lattice volume by X-ray diffraction studies has been shown to occur in hypertonic solutions (33). In addition to this possibility, Gordon and Godt (14) have recently discussed in detail the possible direct effects of hypertonic solutions on the contractile proteins, such as an influence on actomyosin and on ATPase...

G. NIEMEYER AND W. G. FORSSMANN Glycerol Treatment in Muscle and Heart 297
activity. Such a mechanism was first reported by Hasselbach (16), and more recently Portzehl (unpublished, personal communication) has found, in extracted rabbit skeletal muscle, a significant decrease in ATPase activity in hypertonic glycerol-Tyrode's (500-1000 mm).

Certain problems concerning the differences between skeletal and cardiac muscle stressed in a recent paper by one of us (9) now become more prominent. Two main morphological properties may be responsible for the insensitivity of the heart muscle T system to glycerol treatment. First, the T system membrane in heart has been shown to be coated with a 200 A thick basement membrane, which is absent in skeletal muscle. Second, the size of the lumen of the T tubules is much larger in heart muscle than it is in skeletal muscle. Since, after the change to normal Tyrode’s solution, water will flow through the surface and tubular membrane into the fiber, glycerol will slowly diffuse to the bathing solution through the surface membrane and via the T system. The different diffusion rates of water and glycerol as well as a glycerol concentration gradient between the T system and the extracellular fluid immediately after removal of the hypertonic solution may cause extreme swelling and disruption of the tubules in skeletal muscle. In heart muscle the T tubules are much shorter and wider, so an equilibrium is more likely to be reached between the T system and the bathing solution before extreme swelling and disruption. This may be the reason that the T system in heart muscle is mechanically less stressed during osmotic changes than that in skeletal muscle. Not only are there morphological differences between the T systems in skeletal muscle and in heart, but there also exist differences in the physiological response to glycerol treatment as has been shown in this paper.

In the experiments with glycerol in heart muscle, only slight reversible changes of the action potential characteristics were found. These small changes are in the range of normal variability (Weidmann (35), and personal communication). Furthermore, in heart muscle the slope and time constant of hyperpolarizing current pulses did not change with the glycerol treatment, and the amount of applied current for a small voltage displacement remained constant.

The fact that no changes in the shape of a hyperpolarizing step were observed is in agreement with the lack of disruption of the T system, since marked changes were found by Gage and Eisenberg (13) in skeletal muscle where disruption of the T system occurs.

The agreement between the negligible changes in heart muscle with respect to electrophysiological and electron microscope findings after the glycerol treatment is thus confirmed in the present study.

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