J. Membrane Biol. 67, 81-90 (1982)

The Journal of
Membrane Biology

Effect of Chloride Withdrawal on the Geometry of the T-Tubules in Amphibian and Mammalian Muscle

Angela Dulhunty
Department of Anatomy, The University of Sydney, New South Wales, Australia

Summary. The relative chloride permeabilities of the T-tubule membranes in mammalian (rat sternomastoid) and amphibian (toad sartorius) skeletal muscle fibers have been assessed from the change in volume of the T-tubules during chloride withdrawal from fibers exposed to low extracellular chloride concentrations. A 3.5- to 4.2-fold increase in T-tubule volume was found in mammalian fibers, and this was shown to be independent of the composition of the low chloride solution or the nature of the fixative used in preparation for electron microscopy. The increase in T-tubule volume was transient and was inhibited by factors which block chloride conductance, i.e., low pH, 2,4-dichlorophenoxyacetic acid, and nitrate ions. A small increase (1.48-fold) in T-tubule volume was seen in amphibian fibers when chloride ions were replaced by sulphate ions. No increase in volume was observed in amphibian T-tubules when methyl sulphate ions replaced chloride ions. The results support the idea that the chloride permeability of the T-tubule membrane is significantly higher in mammalian fibers than in amphibian fibers.

Key words choride withdrawal · T-tubule geometry · skeletal muscle

Introduction

It has been suggested that the membrane of the transverse (T-) tubules in mammalian skeletal muscle fibers has a high resting conductance which can be largely attributed to chloride permeability (Palade & Barchi, 1977; Dulhunty, 1979). This result is surprising since the T-tubule membrane in amphibian muscle fibers has a low resting conductance (Gage & Eisenberg, 1969) and a low permeability to chloride ions (Hodgkin & Horowicz, 1960; Eisenberg & Gage, 1969). The chloride permeability of the T-system in mammalian fibers was assessed from an analysis of the time course of changes in membrane potential with changes in external potassium and chloride ion concentrations (Dulhunty, 1979) and from the effect of glycerol treatment (Fujino, Yamaguchi & Suzuki, 1961; Howell & Jenden, 1967; Eisenberg & Gage, 1969; Gage & Eisenberg, 1969; Dulhunty & Gage, 1973) on total membrane conductance (Palade & Barchi, 1977; Dulhunty, 1979). Both techniques are open to criticism when used with mammalian muscle. The measurement of the time course of membrane potential changes is complicated by the fact that single fibers are difficult to isolate and the experiments were done on fiber bundles, where diffusion delays between the bulk solution and muscle fiber membrane could have influenced the results. The glycerol-treatment experiments were complicated by the fact that glycerol-treated mammalian fibers are depolarized (Dulhunty, 1979; Davey, Dulhunty & Fatkin, 1980) and the effect of the treatment on the surface membrane is unknown.

A different method has been used in this study to assess the relative permeabilities of the T-tubule membranes in amphibian and mammalian muscle fibers. Foulks, Pacey and Perry (1965) suggested that chloride efflux across the T-tubule membrane, during chloride withdrawal from fibers exposed to low extracellular chloride concentrations, would increase the tonicity within the T-tubule lumen so that the tubule would swell. In support of this hypothesis, T-tubule swelling has been observed in frog fibers exposed to low chloride solutions (Freygang, Goldstein, Hellam & Peachey, 1964; Foulks et al., 1965; Rappoport et al., 1969). The interpretation of this observation has been controversial. Rappoport, Peachey and Goldstein (1969) argued that, in each case, swelling was due either to a decrease in ionic strength or to the presence of sucrose in the T-tubule lumen, and was not a direct result of chloride efflux across the T-tubule membrane. Their argument did not take into account the fact that T-tubule swelling was observed by Foulks et al. (1965) when external sodium chloride was replaced by sodium acetate, i.e., without the addition of sucrose and with a constant ionic strength. The experiments reported here support the hypothesis put forward by Foulks et al. (1965). Muscles were exposed to low chloride solutions with normal tonicity and ionic strength, and the fixation for electron

0022-2631/82/0067-0081 $02.00

Table 1. Solutions

Solution		Na	K	Mg	Ca	Cl	SO_4	HCO_3	Hepes	Glucose	Sucrose
Krebs[a]	*A*	145	3.5	1	2.5	130.5	–	25	2.0	11.0	–
	B	150	3.5	1	2.5	158.5	–	–	2.0	11.0	–
	C	84	3.5	1	8.0	–	52.75	–	2.0	11.0	175.5
Ringer's[b]	*D*	118	2.5	–	1.8	124.1	–	–	3.0	–	–
	E	82	2.5	–	8.0	–	50.25	–	3.0	–	113.0

[a] pH adjusted to 7.4. [b] pH adjusted to 7.2.
NaOH was added to the stock Hepes solution until the pH reached the desired level.
2.5 mM 2,4-D was added to solutions *A*, *B* and *C* where appropriate.
In some cases the pH of the solution was adjusted to 4.4 by adding 2.0 mM Hepes at pH = 4.4.

microscopy was done in the same solution to preserve intracellular volume (Davey, 1973). A significant increase in T-tubule volume was found in mammalian preparations. The swelling was transient and was inhibited by factors that block chloride conductance, i.e., low pH (Hutter & Warner, 1967; Palade & Barchi, 1977), 2,4-dichlorophenoxyacetic acid (Rudel & Senges, 1972), and nitrate ions (Moore, 1969). The results support the chloride withdrawal hypothesis, and the high chloride permeability of the T-tubule membrane in mammalian muscle fibers.

Materials and Methods

The experiments were done in flattened sheets of 50–100 fibers (i.e., 20 to 30 fibers wide and 3 to 5 fibers thick) dissected from the red segment of rat (male Wistar) sternomastoid and toad (*Bufo marinus*) sartorius muscles in a normal (solution *A* or *D*, Table 1) solution at room temperature (usually 20±2° C). The fiber bundles were pinned out, at rest length, in a Sylgard (Dow Corning) lined petri dish, exposed to control or test solutions (Table 1), and subsequently fixed for electron microscopy, in the same solution. The solutions listed in Table 1 were used as vehicles for both glutaraldehyde (or glutaraldehyde + acrolein) and osmium tetroxide, OsO_4, and for washing the preparations after exposure to each fixative (Davey, 1973). This technique has been found to preserve fiber volume and T-tubule volume in hypertonic solutions (Davey & O'Brien, 1978), giving results similar to those obtained with freeze substitution techniques (Franzini-Armstrong et al., 1978). Initial fixation was in 5% glutaraldehyde (or 1% glutaraldehyde + 4% acrolein) for 2 hr at 0–5 °C. The fiber bundle was then washed for 2 hr, with four changes of solution, postfixed in 1% OsO_4 for 2 hr and washed for a further hour. Bundles of 3–5 fibers were peeled from the outside of the preparation and cut into 2- to 3-mm segments suitable for embedding. The tissue was exposed to 0.1 N sodium acetate for 9 min, stained with 2% aqueous uranyl acetate overnight, then dehydrated and embedded in Epon. Thin sections were cut on a ultramicrotome and viewed with a Philips EM 303 electron microscope.

Sampling of T-tubules for Measurement

Ten to 24 fibers were selected from two to three preparations in normal solutions and in the low chloride solutions after appropriate times. Fibers with a sectioned diameter greater than 30 μm were selected to ensure that the section passed through the center of the fiber. This precaution was taken because T-tubule diameter often increased as the tubule approached the fiber surface and tubules sampled from a section grazing the surface would have unrealistically large average dimensions. An area near the center of the fiber was selected, and serial photographs were taken along a group of myofibrils. All T-tubules clearly in triads (i.e., with rows of feet and a terminal cisterna on either side) were measured. When a length of triad greater than 80 nm was sectioned, several measurements, at regular 50-nm intervals, were taken along the T-tubule.

Measurement of T-tubule Short Axis and Junctional Gap

The T-tubule can be described as a thin sac with a short, longitudinally oriented axis and a long, transversely oriented axis which faces towards the sarcoplasmic reticulum, SR. In longitudinal sections of muscle fibers the T-system is most easily identified where it makes junctional contact with the sarcoplasmic reticulum. There is usually one junction per sarcomere (in register with the *Z*-line) in amphibian muscle (Peachey & Schild, 1968) and two junctions per sarcomere (in register with the *A I* junction) in mammalian muscle (Porter & Palade, 1957). The method used to measure the short axis of the T-tubule and the junctional gap at the triad junction is schematically illustrated in Fig. 1. Measurements were confined to T-tubules whose membranes demonstrated a clear bilayer structure. Two measurements were made; d_o, the separation between opposing junctional SR, and d_i, the separation between opposing T-tubular membranes. The measurements were taken from the center of each membrane and corrections were made for the width of the membrane, w, which was estimated to be 8.5 nm in these preparations. The tubule width was equal to d_i-w and the junctional width was equal to $[(d_o - d_i)/2] - w$. The measurements were made using a vernier caliper.

The method just described provides an accurate measure of the T-tubule short axis when triads are cut in the orientation shown in Fig. 1*C*. Since only a few triads were cut in this orientation it was necessary to include data from tubules cut along their length (*see* example in Fig. 2*C*). In this case an error would have been involved in the short axis measurement if the section was not exactly oriented at 90° to the long axis of the tubule cross section, i.e., cut in a plane similar to that shown in Fig. 1*B*. An attempt was made to estimate the error in toad fibers by comparing the average short axis of triads cut exactly through their shortest dimension with the average short axis of all triads. The values were 26.6±4.1 nm (mean±SE; 28 observations) and 24.2±1.7 nm (mean±SE; 407 observations, *see* Table 3), respectively. The average values are not significantly different but the errors are large. A theoretical maximum error can be calculated, since only those triads showing "feet" clearly on both sides of the T-tubule were used for measurements (*see* above). The parallel rows of feet are

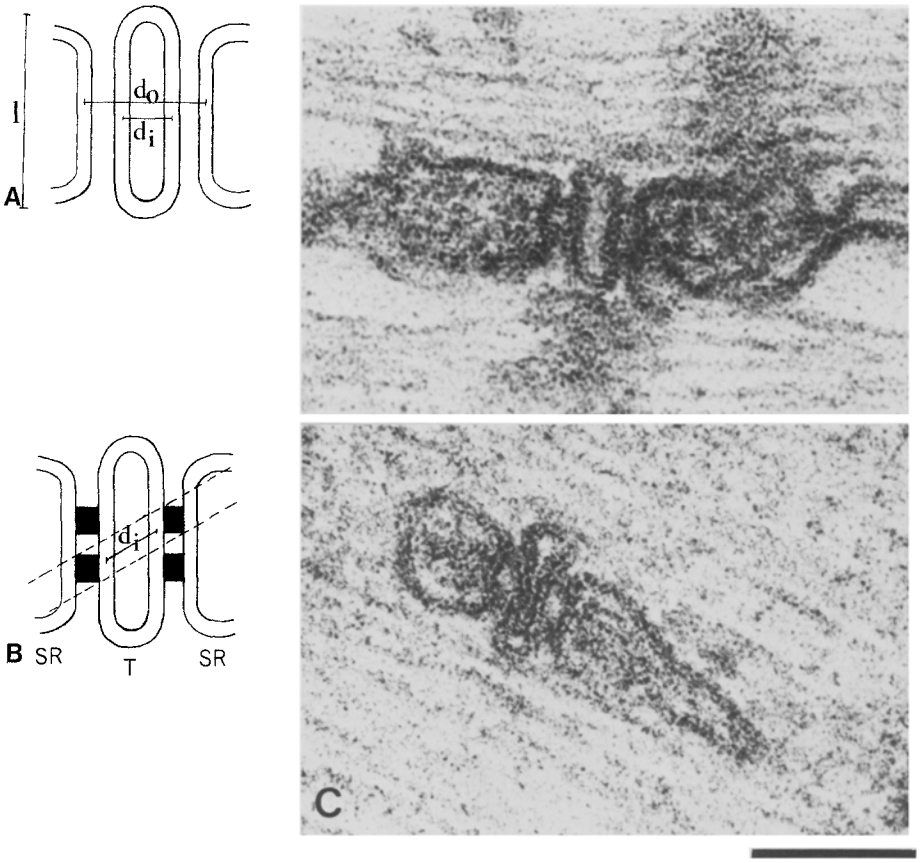

Fig. 1. Methods used to determine the length of the axes of the tubule cross section and the width of the junctional gap. (*A*): A schematic illustration of a triad: d_i is the distance between the center of opposing tubular membranes; d_o is the distance between opposing membranes of the terminal cisternae. Both d_i and d_o are roughly parallel to the longitudinal axis of the fiber. The tubular short axis and junctional gap were calculated from d_i and d_o (*see* text). $\mathscr{L}$ is the distance between the center of opposing tubule membranes and is approximately parallel to the transverse axis of the fiber. The long axis of the tubule is calculated from $\mathscr{L}$ (*see* text). (*B*: A section through the T-tubule, not parallel to the tubular short axis, but with the maximum eccentricity that would allow junctional feet on either side to be included in the section. The percentage error involved in measurement of d_i^1 from tubules sectioned in this manner is calculated as $[w(d_i^1 - d_i)\ 100]/[d_i^1 - w]$ (*see* text). (*C*): Two triads supposedly sectioned through their narrowest dimension to reveal both the short axis and the long axis of the tubule. The upper triad is from a frog sartorius fiber and the lower triad is from a rat sternomastoid fiber

arranged in a regular array so that the width of the foot is 15 nm and is separated from the next foot by a distance of 12 nm (Franzini-Armstrong, 1970). An example of a section that could be oriented at less than 90° to the long axis yet still include feet on both sides of the T-tubule is shown in Fig. 1 *B*. The maximum error would be an overestimate of the short axis by 30% for a tubule with a true axis of 10 nm or 8% for a tubule with a true axis of 50 nm. Since the error is less when the tubule dimensions are greater, the relative swelling seen in low chloride solutions may be underestimated.

Measurement of T-tubule Long Axis

The long axis of the T-tubule is difficult to measure in longitudinal sections. However, it can be seen when the triads have been cut through their narrowest dimension at the median part of the myofibril (Peachey, 1965). The dimensions do depend on the exact plane of section and the selection of appropriate triads is subjective and subject to error. However, an estimate of this dimension was necessary, and the same errors were presumably met in each preparation so that relative changes are meaningful. Examples of triads thought to be cut through their narrowest dimension are shown in Fig. 1 *C*. Three criteria were strictly observed in the selection of such triads:

1. The demonstration of the characteristic butterfly outline, shown in Fig. 1 *C*.
2. The presence of no more than two "feet" bridging the junctional gap between the T-tubule and terminal cisternae (Franzini-Armstrong, 1970).
3. The membrane around the entire T-tubule was cut in a uniform manner with the bilayer clearly visible.

The distance (*see* Fig. 1) was measured and the long axis of the tubule was equal to $\mathscr{L}$–8.5 nm.

Calculation of T-tubule Cross-Sectional Area

The cross-sectional area of the T-tubule was calculated from the measured long and short tubular axes by assuming that the tubule had an elliptical profile. This assumption often appeared to be reasonable; however, some T-tubules had dumbell (Fig. 1 *C*) or square outlines (*see* Fig. 2 *D*). The errors in elliptical assumption were estimated to be of the order of 8–12% (underestimate) for dumbell tubules or as much as 20% (underestimate) for square

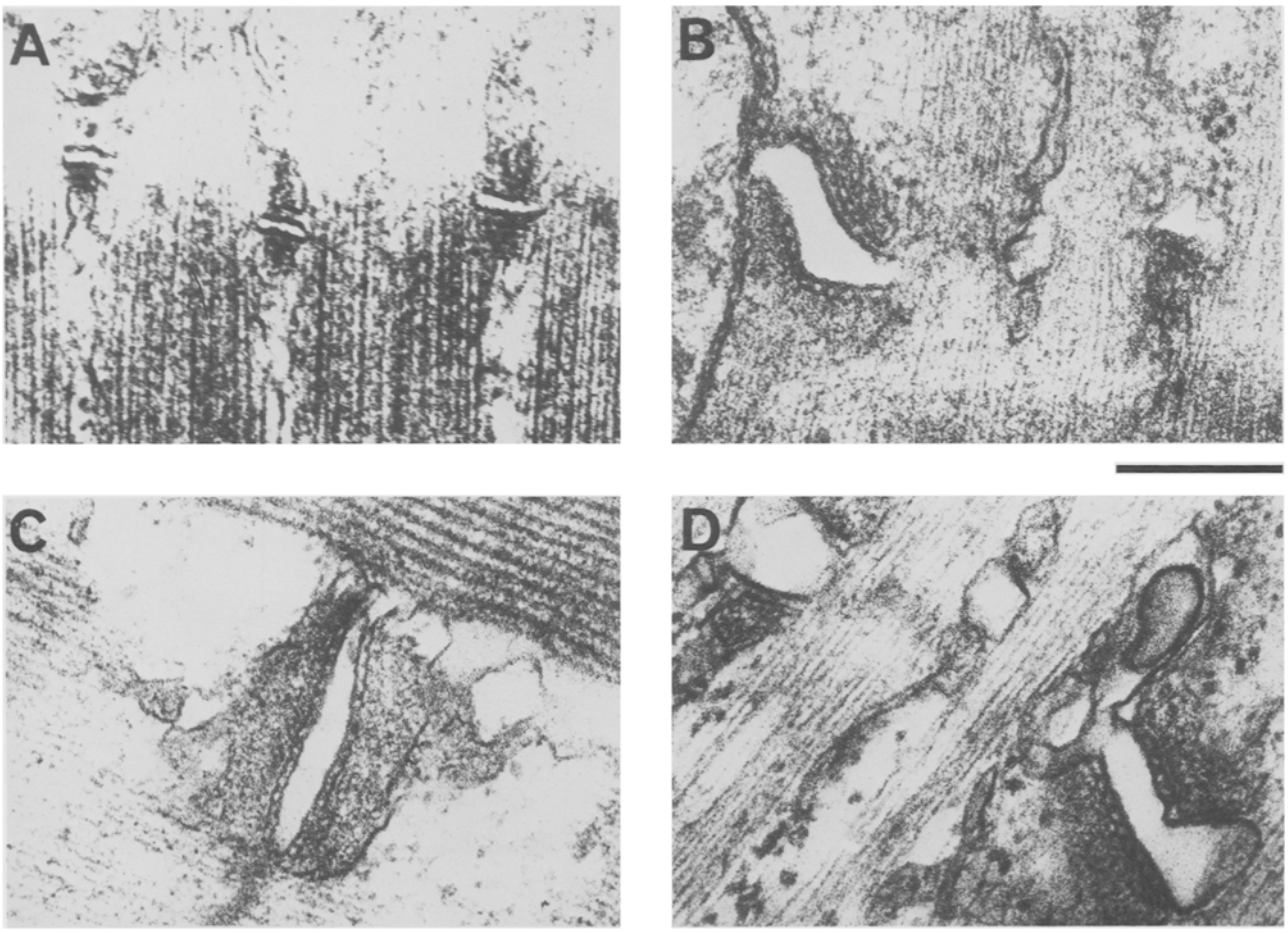

Fig. 2. Electron micrographs showing the effect of chloride withdrawal, from fibers in low extracellular chloride concentrations, on the profile of T-tubules. (*A*): Rat sternomastoid fiber in control solution (solution *B*; Table 1). (*B*): Rat sternomastoid fiber after 15 min in low chloride solution (solution *C*; Table 1). (*C*): Toad sartorius fiber in control solution (solution *D*; Table 1). (*D*): Toad sartorius fiber after 10 min in low chloride solution (solution *E;* Table 1). The calibration bar is 168 nm

tubules. Since tubules swollen during chloride withdrawal often assumed a square outline, the elliptical assumption may have led to an underestimate of the true degree of tubule swelling.

The two major errors in the analysis of the Results, i.e., that arising from the inclusion of T-tubules cut longitudinally (*see* previous section) and that arising from the elliptical assumption, would influence the results in such a way that the calculated increase in the volume of a unit length of T-tubule (during chloride withdrawal) may be less than the true increase in volume.

Measurement of Fiber Volume: Frozen Sections

Fiber volume was measured from frozen sections of fibers in control and test solutions. The bundle of fibers was cut into 3- to 5-mm segments in control or test solutions. The fibers contracted when cut and then relaxed after several minutes. Excess solution was removed from the muscle segment by placing it briefly onto filter paper. The muscle was then rapidly frozen in liquid Freon (precooled in liquid nitrogen). Tissue sections (1 μm thick) were cut on a cryomicrotome, air dried at room temperature, and stained with methylene blue. Two axes of the fiber, at right angles to each other, were measured and fiber cross-sectional area was calculated assuming that the fibers had an elliptical outline. Fiber areas calculated in this way are very similar to areas calculated from electrophysiological analyses (Davey et al., 1980), suggesting that there is not a significant volume change during preparation of the sections.

Results

The experiments reported in this paper were done with rat red sternomastoid fibers and toad sartorius fibers (*see* Materials and Methods). For brevity, these will be referred to as rat and toad fibers. However, the muscles are not necessarily representative of all toad and rat muscles. The surface membrane resistance of slow muscle fibers in amphibia is different from that in twitch fibers (Chandler, Gilly & Hui, 1978), and the rat diaphragm has been found to differ from the sternomastoid and limb muscles (Dulhunty, 1978). The experiments reported in this section were done using sulphate ions (i.e., solutions *C* and *E*) to replace chloride ions, and the preparations were fixed in 5% glutaraldehyde.

Transient swelling of T-tubules was observed dur-

ing chloride withdrawal from both rat and toad fibers. Representative micrographs of normal T-tubules, from fibers in control solutions, and of swollen T-tubules, from fibers in low chloride solutions, are shown in Fig. 2. The normal T-tubules in the rat (Fig. 2*A*) have a narrower and more regular short axis than the normal toad tubules (Fig. 2*C*). The detailed geometry of T-tubules in rat and toad muscles is discussed elsewhere (Dulhunty, 1982). The T-tubule profiles tend to become more square than rectangular in the low chloride solution and the swelling is more noticeable in rat fibers (Fig. 2*A* and *B*) than in toad fibers (Fig. 2*C* and *D*). This effect is in part due to the more regular outline of the rat T-tubule (compared with the variability in toad T-tubules), but is also due to a significantly greater increase in the average dimensions of the rat T-tubules (*see* following sections).

The T-system was the only component of the fiber to be obviously altered during chloride withdrawal. Other structures, i.e., mitochondria, nuclei, sarcoplasmic reticulum, and myofilament lattice, all appeared to be normal. Although specific measurements were not made on these structures, the preservation of a normal appearance, in both toad and mammal, suggests that there were no gross changes in fiber volume, either during preparative procedures or chloride withdrawal, that may have differentially affected tubular volume.

Measurements on Rat Sternomastoid T-tubules

The short and long axes of the T-tubule cross-section and the junctional gap, between the T-tubule and the terminal cisternae of the sarcoplasmic reticulum, were measured. The number of triads in which the T-tubule long axis could be measured (*see* Materials and Methods) were small, and the standard errors on the numbers are large (*see* Table 2). Confidence in the measurement was increased by the fact that the average value of 70 nm measured in the toad (*see* Table 3 below) was similar to the value of 80 nm reported for frog fibers (Peachy, 1965).

Average values for the three measured parameters are listed in Table 2. Both the short and the long axes were significantly ($P < 0.0005$; students t-test) greater than normal in fibers exposed to low chloride solutions for 5 to 60 min. There was a maximum twofold increase in dimensions between 15 and 30 min. After 120 min in the low chloride solution the T-tubules were still swollen, but the dimensions were very much closer to their normal values. There was a small, maintained increase in the width of the junctional gap which was significant ($P < 0.005$, students t-test) at 30 and 60 min.

Table 2. Average values for T-tubule short and long axes in rat sternomastoid fibers exposed to control solution,and from fibers exposed to low chloride solutions for the indicated times[a]

	Number of fibers	Short axis (nm)	Long axis (nm)	Junctional gap (nm)
Control	10	8.5 ± 0.3 (353)	55.3 ± 1.6 (62)	9.2 ± 0.4 (92)
5 min	24	13.7 ± 0.4 (531)	84.8 ± 2.1 (62)	9.9 ± 0.4 (100)
10 min	19	16.4 ± 0.4 (637)	87.8 ± 1.8 (74)	9.5 ± 0.3 (116)
15 min	15	15.1 ± 0.4 (451)	103.0 ± 3.1 (33)	8.4 ± 0.5 (23)
30 min	18	18.0 ± 0.4 (591)	91.5 ± 3.6 (26)	13.2 ± 1.5 (116)
60 min	14	16.9 ± 0.4 (708)	86.1 ± 2.2 (48)	13.1 ± 0.3 (203)
120 min	16	10.9 ± 0.4 (338)	66.9 ± 2.8 (37)	10.2 ± 0.6 (35)

[a] Fibers from two preparations were examined for each exposure time. Results are expressed as mean ± SE, with the number of measurements in parentheses.

The short axis measurements are shown in the histograms in Fig. 3. There was very little scatter in the control data (Fig. 3*A*) where more than 60% of measurements fell below 10 nm and no tubules were greater than 40 nm. The maximum short axis measurement was 34.1 nm. The distribution was very different after 10- to 60-min exposures to the low chloride solution. Fewer than 30% of measurements fell below 10 nm, and 3 to 6% were greater than 40 nm. The increase in tubule dimensions within one fiber was not uniform, and some very swollen tubules were encountered. The maximum short axis measurement was 88.2 nm. The average value for normal T-tubules was 8.5 nm, and this more than doubled to 17.89 nm after 30 min of chloride withdrawal (Table 2).

Measurements on Toad Sartorius T-tubules

The same measurements were made on toad triads, and the average data is given in Table 3. As in the rat, the tubule cross-sectional area was larger than normal in fibers exposed to the low chloride solution. There is an increase in both the short and long axes of the tubule. However, the data in Table 3 differs from that given for the rat in both the time course and magnitude of tubule swelling. The maximum increase in T-tubule dimensions was encounterd 5 to 10 min after exposure to the low chloride solution, and the tubules returned to their normal size between 30 to 60 min. The maximum tubule dimensions in

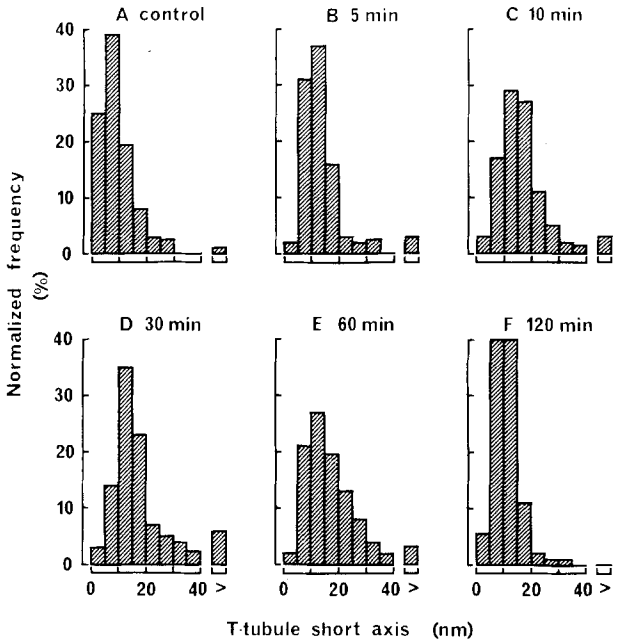

Fig. 3. The distribution of T-tubule short axis measurements in rat sternomastoid fibers in control solution (solution *B;* Table 1) and after the indicated times in low chloride solution (solution *C;* Table 1). The frequency is shown on the vertical axes and the short axis dimension, in nm, on the horizontal axis. The separated segment, labeled >, contains the observations of short axes greater than 40 nm

Table 3. Average values for T-tubule short and long axes in toad sartorius fibers exposed to control solution, and from fibers exposed to low chloride solutions for the indicated times[a]

	Number of fibers	Short axis (nm)	Long axis (nm)	Junctional gap (nm)
Control	7	24.2±1.7 (407)	69.0±3.3 (33)	7.8±0.3 (141)
5 min	10	32.7±1.4 (272)	75.5±2.9 (35)	6.6±0.3 (73)
10 min	7	28.9±1.4 (212)	79.7±2.3 (35)	8.6±0.3 (142)
30 min	12	28.6±1.6 (221)	65.5±5.5 (14)	9.0±0.4 (46)
60 min	11	25.6±1.2 (221)	59.0±3.0 (28)	9.0±0.2 (85)

[a] Two preparations were examined for each exposure time. Results are expressed as mean ± SE, with the number of measurements in parentheses.

the low chloride solution were only 1.35 times greater than normal. The increase in the short axis after 5 min was significant ($P < 0.005$, students *t*-test) and the increase in the long axis after 10 min was significant ($P < 0.005$ — students *t*-test). As in the rat, there was an increase in the width of the junctional gap after 30 to 60 min in the low chloride solution. The short axis measurements are shown in the histograms in

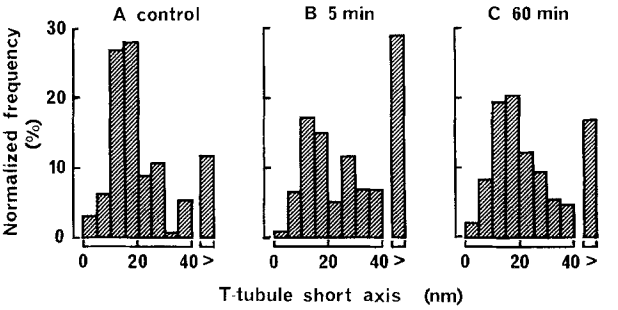

Fig. 4. The distribution of T-tubule short axis measurements in toad sartorius fibers in control solution (solution *D;* Table 1) and after the indicated times in low chloride solution (solution *E;* Table 1). The frequency is shown on the vertical axes and the short axis dimension, in nm, is shown on the horizontal axis. The separated segment, labelled >, contains the observations of short axis greater than 40 nm

Table 4. T-tubule volume, calculated from short and long axis measurements on T-tubules in rat and toad fibers exposed to control solutions and to low chloride solutions for the indicated times

	Time (min)						
	0	5	10	15	30	60	120
Rat T-tubule volume (nm^3)	369	912	1,131	1,222	1,294	1,143	573
Toad T-tubule volume (nm^3)	1,311	1,939	1,809	–	1,471	1,186	–

Fig. 4. In contrast to the rat data there is a broad distribution of values in the control fibers (Fig. 4*A*) with fewer than 10% of measurements falling below 10 nm and more than 10% being greater than 40 nm. The greatest individual measurement was 102 nm. During chloride withdrawal (5 min; Fig. 4*B*) the scatter of data increased, and uneven swelling was once again noted. The number of measurements below 10 nm fell to 7% and the number above 40 nm increased to 27% of the total. The greatest measured dimension was 142 nm. The histogram of data obtained after 10 min in the low chloride solution also shows greater than normal spread of data, although the average value is in fact similar to the control. The mean short axis values increased from 24.2 nm in the control fibers to 32.7 nm after 5 min in the low chloride solution and returned to 25.6 nm after 60 min in the low chloride solution.

T-tubule Volume Changes in Rat and Toad

The data given in Tables 2 and 3 can be used to calculate the volume of a unit length of T-tubule, assuming that the tubules have an elliptical outline. The calculated volumes are listed in Table 4, and relative volumes have been plotted in Fig. 5. The increase

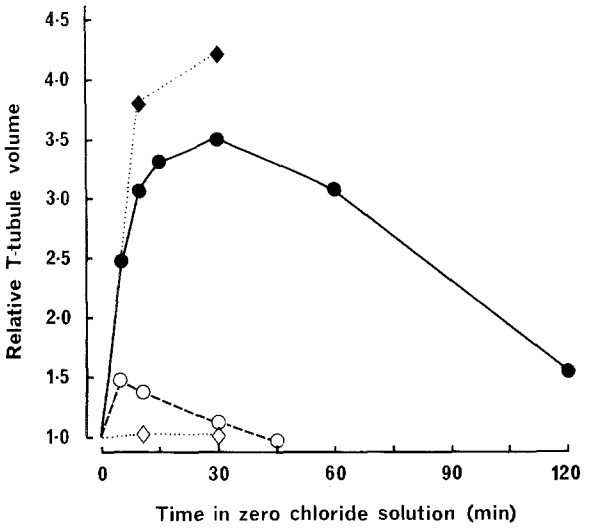

Fig. 5. The relative volume of a unit length of T-tubule (vertical axis) is plotted as a function of time (horizontal axis) after exposure to the low chloride solution. Open symbols – data from rat sternomastoid fibers; filled symbols – data from toad sartorius fibers; circles – fibers fixed in sulphate solutions; triangles – fibers fixed in methyl sulphate solutions. The lines have been drawn through the experimental points

in the volume of rat T-tubules (filled symbols) is seven times greater than that in the toad (open symbols) and is maintained for a very much longer time.

The results presented thus far are consistent with the hypothesis that chloride (or potassium chloride) efflux into the T-system causes swelling and that the chloride permeability of the T-tubule membrane is higher in rat fibers than it is in toad fibers. However, it could also be argued that normal T-tubule volume is altered simply by the removal of chloride ions or by the presence of a divalent anion (SO_4^{2-}) or, as suggested by Rappoport et al. (1969), by the presence of sucrose within the T-tubule lumen, although it would be necessary to postulate that the rat T-system is particularly sensitive to these procedures. The transient nature of T-tubule swelling is inconsistent with this argument as are the results of the following experiments.

Replacement of Chloride Ions with Methyl Sulphate Ions

Some experiments were repeated using a low chloride solution with methyl sulphate as the principal anion (i.e., sodium chloride in the Krebs and Ringer's solutions (*see* Table 1, solutions *A* and *D*) was simply replaced by sodium methyl sulphate) so that the low chloride solution did not contain either sucrose or a divalent anion. Bundles of rat and toad fibers were exposed to this low chloride solution for 10 or 30 min before fixation in 1% glutaraldehyde and 4% acrolein

Table 5. Average values for T-tubule short and long axes in rat and toad fibers exposed to a solution in which sodium chloride was replaced by sodium methyl sulphate[a]

		Rat [mean ± SE (*n*)]	Toad [mean ± SE (*n*)]
10 min	Short axis (nm)	20.60 ± 0.71 (518)	20.18 ± 0.04 (444)
	Long axis (nm)	86.90 ± 2.03 (110)	84.28 ± 1.81 (77)
	Volume (nm^3)	1,406	1,336
30 min	Short axis (nm)	23.05 ± 0.66 (588)	21.15 ± 0.55 (325)
	Long axis (nm)	86.30 ± 1.66 (133)	80.29 ± 2.49 (50)
	Volume (nm^3)	1,562	1,334

[a] The periods of exposure to the low chloride were 10 and 30 min. Measurements were obtained from at least 10 different fibers in each preparation. The volume per unit length of the T-tubule was calculated assuming that the T-tubules have an elliptical outline.

(*see* Methods). The results obtained in this study were very similar to the results already described. Average values for short and long tubular axes in each preparation are given in Table 5 and the calculated T-tubule volumes are shown in Fig. 5 (diamonds). Significant swelling is apparent in the rat fibers (filled symbols) but not in toad fibers (open symbols). There was a small increase in T-tubule volume in the toad fibers but this was not significant. It is possible that more swelling may have been seen if muscles had been fixed at earlier times (i.e., times less than 10 min in the low chloride solution). The similarity between volume changes seen in rat, with either sulphate ion or methyl sulphate ion substitution for chloride ions, suggests that T-tubule swelling is not primarily due to the valency of the anion used to replace chloride or to oddities of the sulphate solution (i.e., the presence of sucrose or a high total calcium ion concentration). The fact that similar results were obtained using either glutaraldehyde or glutaraldehyde + acrolein as primary fixatives shows that T-tubule volume changes did not depend significantly upon the particular fixative used.

Effect of Chloride Blocking Agents on T-tubule Swelling in Low Chloride Solutions

Rat muscle fibers were exposed to low chloride solutions under conditions that are known to inhibit surface membrane chloride conductance in mammalian muscle; i.e., low pH (Hutter & Warner, 1967; Palade & Barchi, 1977), 2,4-dichlorophenoxyacetic acid (2,4-D; Rudel & Senges, 1972; Palade & Barchi, 1977), and nitrate ions (shown to block chloride conductance in frog muscle; Moore, 1969).

Table 6. Average values for T-tubule short and long axes in rat sternomastoid fibers exposed to control solutions and to low chloride solutions having low pH or containing 2,4-dichlorophenoxyacetic acid (2,4-D)[a]

	pH = 4.4		2,4-D (2.5 mM)	
	Control	Low Cl	Control	Low Cl
Short Axis				
Mean (nm)	9.5	12.7	7.0	8.6
± SE	±0.4	±0.5	±0.2	±0.2
(*n*)	(207)	(415)	(223)	(217)
Long Axis				
Mean (nm)	71.3	74.0	71.3	74.2
± SE	±2.8	±4.0	±3.8	±2.8
(*n*)	(29)	(31)	(25)	(27)
Volume per unit length nm^3	531.0	738.1	392.0	501.2

[a] The control preparations were exposed to control solutions at pH = 4.4 or containing 2.5 mM 2,4-D for 10 min before fixation in the same solution. Other preparations were exposed to control solution at pH = 4.4, or containing 2.5 mM 2,4-D, for 10 min and then exposed to the low chloride solution at pH = 4.4, or containing 2.5 mM 2,4-D, for a further 10 min before fixation in the final solution (2,4-D). Results are expressed as mean ± SE with the number of measurements in parentheses. The volume was calculated assuming that the T-tubules have an elliptical outline.

Fibers were fixed after exposure to normal Krebs or sulphate Krebs solutions with pH = 4.4 or containing 2.5 mM 2,4-D (the sequence of solution changes is described in Table 6). The average data obtained from these preparations is given in Table 6. In both instances the control T-tubule dimensions were different from the normal values (*see* Table 4 above). The short axis was the most sensitive to the procedures and was 10% larger than normal in the low pH solution ($P < 0.01$; students *t*-test) and 18% smaller than normal ($P < 0.0005$; students *t*-test) in the presence of 2,4-D. Low pH and 2,4-D significantly reduced the degree of T-tubule swelling seen during chloride withdrawal (compare data in Tables 4 and 6). However, there was still a significant increase in the T-tubule dimensions after 10 min in the low chloride solution, and the volume, per unit length, was 1.4 times control with low pH, 1.3 times control in the presence of 2,4-D. These values can be compared with a 3.5-fold volume increase (*see* Fig. 5) in the absence of chloride blocking agents. The residual swelling seen in low pH solutions and with 2,4-D (*see* Table 6) suggests either that chloride permeability is not totally blocked (and that low pH is a less efficent chloride blocking agent than 2,4-D), or that there is a small effect of sucrose (or SO_4^{2-}) on T-tubule volume.

One preparation was fixed after 30 min exposure to a solution in which chloride ions had been replaced by nitrate ions. The T-tubule short axis was 8.3 ± 0.3 nm (mean ± SE; 349 observations), and the long axis was 69.1 ± 1.5 nm (mean ± SE; 118 observations). The average volume was 450 nm^3, or 1.22 times the normal volume. The simplest interpretation of this observation is that chloride is unable to enter the T-system through channels blocked by nitrate ions and T-tubule swelling did not occur.

These results show that T-tubule swelling is primarily dependent upon a high chloride conductance and is indicative of chloride withdrawal across the T-tubule membrane into the tubule lumen.

Table 7. Fiber cross-sectional areas measured from frozen sections of mammalian muscles in control solutions and after indicated times in low chloride solutions[a]

	Control	5 min	Control	30 min
Mean m^2	1,994	2,132	1,709	1,881
± SE	±34	±41	±38	±34
(*n*)	(697)	(594)	(558)	(416)

[a] The results are expressed as mean ± SE with the number of observations in parentheses. In each case a bundle of fibers was split in half and one half exposed to the low chloride solution while the other half was retained in the control solution.

Effect of Chloride Withdrawal on Fiber Volume in the Rat

Foulks et al. (1965) suggested that, if a Donnan equilibrium existed across the muscle surface membrane, potassium efflux would follow a reduction in the external chloride concentration, to maintain a constant [K] × [Cl] product inside and outside the cell. Thus T-tubule swelling could be principally due to potassium withdrawal. Under these conditions the low chloride solution would be hypertonic and the cell would shrink. Therefore, cell volume can be used to determine whether a Donnan equilibrium is maintained. Observations on the appearance of the fiber (*see* above) suggested that gross changes in fiber volume did not occur during chloride withdrawal. However, it is possible that fiber volume may have been altered during fixation (Eisenberg & Mobley, 1975) so that initial volume changes were not preserved. Therefore fiber volume was estimated from the cross-sectional areas of fibers in frozen sections (*see* Table 7). Once again no shrinkage was apparent. In fact, a small, but significant ($0.0005 < P < 0.001$, student *t*-test), increase of 10% in fiber volume is apparent in fibers frozen after 30 min in the low chloride solution. Electrophysiological evidence (Hodkin & Horowicz, 1960; Dulhunty, 1979) also suggests that the internal potassium concentration is not radically altered by lowering the external potassium concentration. Some potassium may enter the T-tubule with chloride to main-

tain electrical neutrality. However, other ions may also be involved.

It is interesting to consider whether the increase in fiber volume could be due to the increase in T-tubule volume. The T-system normally occupies 1% of the fiber volume in rat pharyngeal arch muscles (Hinrichsen & Dulhunty, 1982) and other rat muscles (Davey & O'Brien, 1979). If the increase in volume of a unit length of T-tubule reflects an increase in the volume of the fiber occupied by the T-system, T-tubule swelling could account for a 3.5% increase in fiber volume.

Discussion

The transient nature of T-tubule volume change during chloride withdrawal, its independence of the anion used to replace chloride, and its dependence on normal chloride conductance show that swelling is primarily due to chloride efflux across the T-tubule membrane and is not an artifact of either the presence of sulphate ions or of sucrose in the bathing solution, as suggested by Rappoport et al. (1969). The same authors cite the lack of T-tubule swelling in solutions made hypertonic by addition of excess sodium chloride and in low external potassium solutions as evidence that T-tubule volume is not influenced by potassium chloride efflux. However, subsequent work has shown that the preservation of T-tubule volume during preparation for electron microscopy is critically dependent on the fixation techniques (Davey, 1973) and that hypertonic sodium chloride does in fact lead to significant T-tubule swelling in amphibian (Somlyo, Shuman & Somlyo, 1977; Franzini-Armstrong et al., 1978) and mammalian (Davey & O'Brien, 1978) muscles. The effect of low external potassium concentration on T-tubule volume remains to be reinvestigated.

The results support previous electrophysiological data (Hodgkin & Horowicz, 1960; Eisenberg & Gage, 1969; Palade & Barchi, 1977; Dulhunty, 1979) showing that chloride conductance in the T-tubule membrane is small in amphibian muscle and large in mammalian muscle. The relative chloride permeabilities of the T-tubule membranes have not been calculated since the relation between T-tubule volume and membrane permeability to any solute is complex because of the fact that the T-system is not a closed compartment and there is bulk water flow and solute diffusion along the length of the tubule to the extracellular space (*see* Barry, 1973). The conductance of the T-tubule membrane is one factor determining the electrical space constant of the T-system. The higher chloride conductance in the rat must significantly reduce its tubular space constant. The space constant in the sternomastoid fibers is probably reduced even further by the small diameter of the tubules (*see* Table 2).

The time dependence of T-tubule volume changes during chloride withdrawal has not been followed in previous studies. The slow rates of volume increase and decrease in rat was unexpected since the membrane potential changes (which reflect bulk ion concentrations) are complete within 15 to 20 min (Dulhunty, 1978). Slow fixative diffusion into the narrow rat T-tubule would, if anything, shift the curve shown in Fig. 5 to the left and apparently increase the rate of tubule swelling and recovery. Slow tubule fixation in the rat may hide a transient increase in volume at early times, corresponding to the time of the peak volume increase seen in the toad. If this were the case, the true volume increase in rat may be several times larger than that measured in these experiments. However, it is also possible that diffusion of chloride from the rat T-system may be slowed because the T-tubule dimensions are normally smaller than in the toad (*see* Tables 2 and 3). The greater volume increase in the rat may in fact lead to partial isolation of areas of the T-system (Dulhunty & Gage, 1973), thus restricting bulk water flow and chloride diffusion out of the tubules. The chloride pump (Dulhunty, 1978) might also act to prolong the volume change in the rat T-system. If the pump was located in the T-tubule membrane it would transport chloride ions trapped in the T-system back into the sarcoplasm and thus maintain a small intracellular chloride concentration. With low chloride concentrations outside the cell, a small intracellular concentration, possibly confined to an area adjacent to the T-tubules, would have little effect on the membrane potential but could, by diffusion back into the T-tubules, maintain an excess number of ions within the tubular lumen and hence maintain the T-tubule volume.

The author wishes to thank Professor P.W. Gage for his helpful discussion and Mrs. M. Wandracz for her assistance with electron-microscopy and with measurements of T-tubule dimensions. I would like to thank Mrs. N. Capes and Mr. G. Williams for their technical assistance. The work was supported by a grant from the Muscular Dystrophy Association of America.

References

Barry, P.H. 1973. An approximate analysis of the swelling of the transverse tubular system of a muscle fibre following loading of the fibre with a permeant solute such as glycerol. An appendix to Dulhunty & Gage. *J. Physiol. (London)* **234**:373–408

Chandler, W.K., Gilly, W.F., Hui, C.S. 1978. Electrical properties of amphibian slow muscle fibres. *In:* Biophysical Aspects of Cardiac Muscle. pp. 31–43 Academic Press, New York

Davey, D.F. 1973. The effect of fixative tonicity on the myosin filament lattice volume of frog muscle fixed following exposure to normal or hypertonic Ringer. *Histochem. J.* **5**:87–104

Davey, D.F., Dulhunty, A.F., Fatkin, D. 1980. Glycerol treatment in mammalian skeletal muscle. *J. Membrane Biol.* **53**:223–233

Davey, D.F., O'Brien, G.M. 1978. The sarcoplasmic reticulum and *T*-system of rat extensor digitorum longus muscles exposed to hypertonic solutions. *Aust. J. Exp. Biol. Med. Sci.* **56**:409–419

Dulhunty, A.F. 1978. The dependence of membrane potential on extracellular chloride concentration in mammalian skeletal muscle fibres. *J. Physiol. (London)* **276**:67–82

Dulhunty, A.F. 1979. Distribution of potassium and chloride permeability over the surface and *T*-tubule membranes of mammalian skeletal muscle. *J. Membrane Biol.* **45**:293–310

Dulhunty, A.F. 1982. Heterogeneity of T-tubule geometry in vertebrate skeletal muscle fibers. *J. Membrane Biol. (Submitted)*

Dulhunty, A.F., Gage, P.W. 1973. Differential effects of glycerol-treatment on membrane capacity and excitation-contraction coupling in toad sartorius fibres. *J. Physiol. (London)* **234**:373–408

Eisenberg, R.S., Gage, P.W. 1969. Ionic conductances of the surface and transverse tubular membranes of frog sartorius fibres. *J. Gen. Physiol.* **53**:279–297

Eisenberg, B.R., Mobley, B.A. 1975. Size changes in single muscle fibres during fixation and embedding. *Tissue Cell* **7**:383–387

Foulks, J.G., Pacey, J.A., Perry, F.A. 1965. Contractures and swelling of the transverse tubules during chloride withdrawal in frog skeletal muscle. *J. Physiol. (London)* **180**:96–115

Franzini-Armstrong, C. 1970. Studies of the triad: 1. Structure of the junction in frog twitch fibres. *J. Cell. Biol.* **47**:489–517

Franzini-Armstrong, C., Heuser, J.E., Reese, T.S., Somlyo, A.P., Somlyo, A.V. 1978. T-tubule swelling in hypertonic solutions: a freeze substitution study. *J. Physiol. (London)* **283**:133–140

Freygang, W.H., Goldstein, D.A., Hellam, D.C., Peachey, L.D. 1964. The relation between the late after potential and the size of the transverse tubule system of frog muscle. *J. Gen. Physiol.* **48**:235–263

Fugino, M., Yamaguchi, T., Suzuki, K. 1961. 'Glycerol effect' and the mechanism linking excitation of the plasma membrane with contraction. *Nature (London)* **192**:1159–1161

Gage, P.W., Eisenberg, R.S. 1969. Capacitance of the surface and transverse tubular membrane of frog sartorius muscle fibres. *J. Gen. Physiol.* **53**:265–277

Hinrichsen, C., Dulhunty, A.F. 1982. The contractile properties, histochemistry, ultrastructure and electrophysiology of the cricothyroid and posterior cricoarytenoid muscles in the rat. *Muscle Cell Motility (in press)*

Hodgkin, A.L., Horowicz, P. 1960. The effect of sudden changes in ionic concentration on the membrane potential of single muscle fibres. *J. Physiol. (London)* **153**:370–385

Howell, J.N., Jenden, J.D. 1967. T-tubules of skeletal muscle fibres: Morphological alterations which interrupt excitation-contraction coupling. *Fed. Proc.* **26**:533

Hutter, O.F., Warner, A.E. 1967. The pH sensitivity of the chloride conductance of frog skeletal muscle. *J. Physiol. (London)* **189**:403–425

Moore, L.E. 1969. Anion permeability of frog skeletal muscle. *J. Gen. Physiol.* **54**:33–52

Palade, P.T., Barchi, P.L. 1977. Characteristics of chloride conductance in muscle fibres of the rat diaphragm. *J. Gen. Physiol.* **69**:325–342

Peachey, L.D. 1965. The sarcoplasmic reticulum and transverse tubules of the frogs sartorius. *J. Cell. Biol.* **25**:209–230

Peachey, L.D., Schild, R.F. 1968. The distribution of the T-system along the sarcomeres of frog and toad sartorius muscles. *J. Physiol. (London)* **194**:249–258

Porter, K.R., Palade, G.E. 1957. Studies of the endoplasmic reticulum: III. Its form and distribution in striated muscle cells. *J. Biophys. Biochem. Cytol.* **3**:269–300

Rappoport, S.I., Peachey, L.D., Goldstein, D.A. 1969. Swelling of the transverse tubular system in frog sartorius. *J. Gen. Physiol.* **54**:166–177

Rudel, R., Senges, J. 1972. Experimental myotonia in mammalian skeletal muscle: Changes in membrane properties. *Pfluegers. Arch.* **331**:324–334

Somlyo, A.V., Shuman, H., Somlyo, A.P. 1977. Elemental distribution in striated muscle and the effects of hypertonicity: Electron probe analysis of cryosections. *J. Cell. Biol.* **74**:828–857

Received 29 June 1981; revised 2 December 1981