Some Effects of Hypertonic Solutions on Contraction and Excitation-Contraction Coupling in Frog Skeletal Muscles

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ABSTRACT In frog fast skeletal muscle, we find a decline of twitch, tetanus, and maximum K and caffeine contracture tensions as tonicity of the bathing solution is increased. The decline of tension independent of the method of producing contraction indicates that the major effect of hypertonicity is directly on contractile tension probably because of the increased internal ionic strength. However, there is some apparent disruption of excitation-contraction (E-C) coupling in solutions made three times the normal tonicity (3T solutions) since: (a) in 3T solutions tetanic and K contracture tensions decline to zero from a value near the average maximum caffeine contracture tension at this tonicity (10% of 1T tetanic tension). At this time, caffeine contractures of 10% of 1T tetanic tension can be elicited; (b) once the K contracture tension has declined, elevated $[Ca^{++}]_o$, 19.8 mM, restores K contracture tension to 13% of 1T tetanic tension. This probable disruption is not caused by changes in mechanical threshold since in 2T solutions the mechanical threshold is shifted by 12 mv in the hyperpolarizing direction. This is consistent with neutralization of fixed negative charges on the inside of the membrane. The repriming curve is also shifted in the hyperpolarizing direction in 2T solutions. Shifts of the repriming curve coupled with membrane depolarizations in 3T solutions (about 20 mv) may produce loss of repriming ability at the resting potential and disruption of E-C coupling.

It has been known for over 60 years that hypertonic solutions can decrease contractile tensions in skeletal muscles (Overton, 1902; Ernst, 1926) and that cells may be excitable under conditions where tension is vanishingly small (Demoor and Philippon, 1909; Ernst, 1926). Hodgkin and Horowicz (1957) stimulated renewed interest in the effects of hypertonic solutions on excitation-contraction (E-C) coupling in skeletal muscle by showing that bathing a muscle fiber in solutions made 2.5 times the normal tonicity of Ringer solution abolished twitch tension while leaving the action potential virtually unaffected. They also found that tetanic tension was about one-third of that at

normal tonicity. Howarth (1958) showed that stimulation of a frog sartorius muscle bathed in a solution three times the normal tonicity increased the resistance to stretch. This suggested to him that the link between excitation and contraction is intact in hypertonic solutions, but that the speed of contraction is much lower so that the muscle is not able to extend the series elastic element sufficiently in a twitch for tension to be measured. Podolsky and Sugi (1967) using a skinned fiber preparation activated by calcium showed that the velocity of shortening is about 10 times slower if the muscle has been bathed before skinning in a solution which is three times the normal tonicity. Also April et al. (1968) showed that the tension produced by the injection of a fixed amount of calcium into a single crayfish muscle fiber declined steeply as the tonicity of the external solution was increased. On the other hand, Caputo (1966) found that solutions of 1.9 times the normal tonicity potentiated the caffeine contracture while markedly decreasing K contracture tension. On the basis of this and other evidence (Fujino and Fujino, 1964; Fujino, Yamaguchi, and Suzuki, 1961) it was suggested that hypertonic solutions exerted their effect both on the contractile proteins and on the coupling between excitation and contraction.

This study was undertaken to elucidate further the effects of hypertonic solutions on contraction and E-C coupling.

A preliminary report of these results has appeared (Godt, Gordon, and Woodbury, 1969).

MATERIALS AND METHODS

Preparation and Dissection

Small bundles of skeletal muscle fibers from the American frog, *Rana pipiens*, were used to minimize the diffusion delay accompanying solution changes. The toe muscle, extensor longus digiti IV, from small frogs was used in experiments in which the small, but usually not negligible, slow fiber population did not introduce uncertainties in the results. However, a pure population of fast fibers was needed in the study of the mechanical threshold and so small bundles of from 2 to 40 fibers were dissected from the semitendinosus muscle. The fibers were taken from the region opposite the nerve entry to eliminate all the slow fibers which have lower mechanical thresholds for contraction with respect to potassium concentration (A. C. Kirby, private communication). In several experiments both tension and potential were measured on the same bundle of fast fibers. However, larger bundles (up to 40 fibers) were better for microelectrode studies and smaller bundles of from 2–20 fibers were preferable for the tension studies. All muscles were allowed to stand for 30–60 min after dissection to allow damaged muscles to be identified and discarded.

Muscles in good condition were transferred to the Lucite and wax perfusion chamber for stimulation, solution changing, tension recording, and microelectrode recording. Stimulation was achieved through two platinum black, longitudinally oriented electrodes. Stimulus isolation was achieved using a low impedance isolation

transformer. This transformer was driven by the output of a Tektronix 161 pulse generator through an emitter follower circuit for better impedance matching. Tension was measured using an RCA 5734 transducer in a bridge circuit. A dummy transducer (5734), mounted in the same heat sink, was placed in the opposite arm of the bridge to decrease the thermal drift. Membrane potentials were measured by the standard microelectrode technique with high impedance preamplifiers and calomel half-cells. The 3 M KCl used to fill the microelectrodes had a pH of 2 to minimize tip potentials. In the experiments in which action potentials were measured, chlorided silver wires were used in place of the calomel half-cells. Tension and potential were displayed on both a Sanborn model 7702A recorder and a Hewlett-Packard model 132A oscilloscope with camera. Solutions were perfused through the chamber using gravity feed and suction draining. The solution in the chamber could be changed in approximately 2 sec. 10 lines with valves and variable flow resistances allowed for up to 10 solutions to be mounted at one time for possible perfusion.

All experiments were done at room temperature (20–26°C).

Solutions

Table I lists the constituents of the major solutions used in these experiments. Other solutions can be deduced from the listed ones by methods described below.

We found early in our experiments that small changes in tonicity would cause large changes in the tension generated by the muscle, hence much care had to be taken to ensure the proper tonicity for each solution. Tonicities were routinely measured using an Advanced Instruments Inc. (Newton Highlands, Mass.) freezing point osmometer. The tonicity of our normal Ringer solution was 235 milliosmols/kg. Tonicities of other solutions, designated 2T, 2.5T, and 3T, were adjusted to 2, 2.5, and 3 times the normal tonicity, either by increasing the concentration of normal

TABLE I
COMPOSITION OF SOLUTIONS

The composition of the major solutions used is listed. The concentration of the constituents is in mM. Curare (10^{-6} g/ml) was added routinely to each solution. The Tris buffer consisted of 2 mM Tris titrated to pH 7.2 with approximately 1.75 mM HCl. The A in KA refers to the impermeant anion whether propionate, methylsulfate, or isethionate. See text for method of determining composition of other solutions used. Tris is tris(hydroxymethyl)aminomethane.

Solution	NaCl	KCl	CaCl ₂	Tris	Sucrose	KA	Approximate osmolality
							milliosmols/kg H ₂ O
1T	117.4	2.5	1.8	2			235
1TK'			1.8	2		120	
2T	240	5	1.8	2			460
2TK'			1.8	2		245	
3TNa'	360	7.6	1.8	2	30.8		710
3TS	117.4	2.5	1.8	2	414		710
3TK'		2.56	1.8	2		358	710

Ringer constituents, primarily NaCl, or by adding an appropriate amount of sucrose, or both.

In an attempt to decrease the time-dependent effects in the hypertonic solutions, $[K^+]_o[Cl^-]_o$ products were kept constant to minimize transmembrane fluxes of KCl. This was possible for solutions made hypertonic with NaCl, but not sucrose since with the elevation in $[K^+]_i$ and $[Cl^-]_i$ that accompanies the increased tonicity, one must elevate both $[K^+]_o$ and $[Cl^-]_o$ by the same factor in the normal "resting solution" to keep constant both (a) the $[K^+]_o[Cl^-]_o$ product at the higher value consistent with changes in $[K^+]_i$ and $[Cl^-]_i$ and (b) the equilibrium potential for K and Cl. For the Tris-buffered Ringer (tris (hydroxymethyl) aminomethane) $[K^+]_o \times [Cl^-]_o = 313$ (mm)². When $[K^+]_o$ was elevated in 1T solution, $[Cl^-]_o$ was decreased by substituting KA for KCl where A refers to an impermeant anion (see below). In the hypertonic solutions the intracellular concentrations of K and Cl increase in approximate proportion to the tonicity (see Blinks, 1965). Thus, in 2T the $[K^+]_i$ and $[Cl^-]_i$ both doubled and $[K^+]_i \times [Cl^-]_i = 4 \times 313 = 1252$ (mm)². Similarly in 3T, $[K^+] \times [Cl^-] = 9 \times 313 = 2817$ (mm)². The compositions of the 2T solutions of varying $[K^+]$ were determined from three constraints: (a) $[K^+]_o$ set to the desired value, (b) $[K^+]_o \times [Cl^-]_o = 1252$ mm², and (c) osmolality = 460 milliosmols/kg H₂O. Thus as $[K^+]$ was increased, $[Na^+]$ was decreased. The difference in osmotic coefficients between Na and K was neglected.

An impermeant anion was substituted for chloride to adjust the $[K^+][Cl^-]$ product. Several different anions have been used, including sulfate (Hodgkin and Horowicz, 1959), propionate (Reuben et al., 1963), methylsulfate (Hutter and Noble, 1960), and isethionate. Sulfate binds calcium and hence is not very attractive as a chloride substitute. Our initial experiments were done with propionate. Methylsulfate was also used but all sources were contaminated with sulfate or carbonate. These contaminants could be precipitated and removed but only in the case of potassium methylsulfate obtained from Eastman Kodak (Eastman Organic Chemicals) was this practical because of the amount of sulfate present. Thus in our later experiments sodium isethionate was used, rather than sodium methylsulfate, because it was available without sulfate contaminants.

Elevated concentrations of calcium and magnesium were used in several experiments. To keep the tonicities constant, excesses of these ions were substituted for either sucrose, when it was present, or sodium. Calcium propionate was used to keep $[K^+]_o \times [Cl^-]_o$ constant. Mg was added as MgCl₂.

Phosphate was used as a buffer in the initial experiments. However, a white precipitate was seen in elevated Ca solutions, presumably CaHPO₄. This was avoided by using Tris as a buffer (Eastman Organic Chemicals). Caffeine (Eastman Organic Chemicals) was added to the proper solution. Since the amount of added caffeine was small, no osmotic compensation was required.

Procedure for Mechanical Threshold and Repriming Experiments

In both the mechanical threshold and repriming experiments, a muscle was subjected to all the elevated potassium concentrations at one tonicity before being transferred into solutions of a different tonicity. At each tonicity the first and last contractures

were with the solution in which all the Na had been replaced with K (1TK' or 2TK'). In both types of experiments $[K^+]_o$ was increased monotonically. Initial contractures were done with either 1T or 2T solution. Tonicity was elevated with added ionic constituents, not sucrose (see Methods). Although Na was present, little or no twitching was observed visually or in the tension record during the perfusion with elevated $[K^+]_o$.

In the mechanical threshold experiments the contracture tension was measured during perfusion with the elevated $[K^+]_o$ solution at the appropriate tonicity. The muscle bundle was bathed in a solution of normal $[K^+]_o$ for that tonicity for 15 min before the next contracture.

The procedures used in the experiments on the effects of tonicity on the repriming of the contractile system were similar to those of Hodgkin and Horowicz (1960 *a*). The repriming of the contractile system was tested by the contracture in response to the 1TK' or 2TK' solutions. The duration of the exposure to the test contracture solution was 30 sec. After this test contracture, the muscle bundle was returned to solutions of different potassium concentrations to set the membrane potential during the recovery period before the next test contracture. The recovery period between test contractures was 2 min. Recovery in the solutions with normal $[K^+]_o$ at both 1T and 2T was more than 95% complete within 2 min. No attempt was made to maximize the recovery times in the elevated $[K^+]_o$ solutions.

RESULTS

Relationship between Tension and Tonicity

Twitch, tetanus, and peak K contracture tensions were measured as functions of bathing fluid tonicity. Twitch and tetanus were measured during supra-maximal stimulation of curarized muscle. The peak K contracture tension was measured while the muscle was perfused by a solution in which all Na^+ had been replaced with K^+ . The average data from many experiments are plotted in Fig. 1. The ordinate is the fraction of the tetanic tension at normal tonicity. The abscissa is the relative tonicity. For tonicities at and above 2T, the plotted tension was that measured after 2 min in the particular solution. This time was chosen because the muscle volume change is complete by this time. This was verified by observation of muscle bundle diameter. The standard deviations are shown in Fig. 1 for the tetanic values at 2T and 3T.

It can be seen in Fig. 1 that tension, no matter how elicited, decreases as tonicity is increased. Peak K contracture tension declines much less rapidly than do tetanic or twitch tensions at intermediate tonicities. In some experiments K contracture tension increases at 2T. This presumably occurs because K contractures are more synchronous in 2T than 1T. In 1T solutions the phasic nature of K contracture combined with the diffusion delay produces an asynchronous activation and a peak K contracture tension which approaches tetanus tension only in bundles of two or three fibers. The contraction speed is slower and the diffusion delay is decreased (due to bundle

shrinkage) in 2T so that the peak K contracture tension approaches tetanus for the size of bundles used. Even so, the peak K contracture tension at 2T is only about half of the tetanic tension at 1T.

The tetanic tension in the solutions of tonicity at or below 2T was usually stable over a period of up to an hour. In 3T solutions tetanic tension declined with time as shown in Fig. 2. The time course of tension decline was investigated in 23 experiments. In four cases, tetanic tension changed by less than 20% in 10 min. In 19 cases, tension fell more than 20% in 10 min. In 4 of

FIGURE 1. Relative twitch, tetanus K contracture, and caffeine contracture tension as a function of solution tonicity. Average twitch (circles), tetanus (triangles), peak K contracture (squares), or maximum reversible caffeine contracture (inverted triangles) tensions are plotted against the relative tonicity of the external solution. The tensions are normalized to the tetanic tension at normal tonicity, 235 milliosmols/kg. The tonicities are expressed relative to this normal value. The standard deviations on the relative tetanic tensions at 2T and 3T are shown. In all cases except the 2.5T points, the standard error of the mean for each point is less than the size of the symbol used. The curves were fit to the points by eye. The maximum reversible caffeine contracture tensions are the maximums of the contracture tension vs. caffeine concentration curves for 1T, 2T, and 3T solutions plotted in Fig. 3. The maximum occurred at caffeine concentrations above 20 mM, 5 mM, and 5 mM for the 1T, 2T, and 3T solutions, respectively.

these 19 cases, tension was immeasurably small after 10 min. In the majority of experiments in which the muscle was exposed to 3T solutions for longer than 30 min, tension was too small to be measured.

Treatment with hypertonic solutions was reversible if the exposure was less than 20 min in 3T solutions and 1 hr in 2T solutions. After exposure to 3T solutions for 20 min, tetanic tension recovered to an average of 89% of the initial value. The decline in twitch tension was greater than that in tetanus tension after exposure of the muscle to hypertonic solutions thus increasing the tetanus/twitch ratio.

Speed of contraction decreased dramatically with increasing tonicity. The

average contraction times (time to peak tension in a twitch) were 33, 80, 205 msec, and 2.5 sec with tonicities of 1T, 2T, 2.5T, and 3T, respectively.

Sucrose and NaCl hypertonic solutions have different effects on the size of the transverse tubule (Freygang, Rapoport, and Peachey, 1967). Therefore the tensions produced in 3T solutions made hypertonic with sucrose and NaCl were investigated. No significant differences were found in the extent of block at 2 min or the time course of decline. However, variability between muscles was so great that small differences may have been missed.

FIGURE 2. Time course of tetanic tension decline in 3T solutions. The tension is normalized to the value at 1 min in the hypertonic solution. The time is measured from the beginning of 3T perfusion. Filled symbols are the K contracture tension, expressed as a fraction of 1 min tetanic tension.

Transmembrane Potentials in 3T Solutions

Membrane potential declined by an average of about 20 mv in the muscles bathed in 3T solutions. This depolarization occurred even if all of the Na^+ was replaced with choline $^+$ or Tris $^+$. Despite this depolarization many but not all fibers remained excitable as measured with intracellular electrodes. Even when tetanic tension had disappeared, action potentials could still be recorded from many cells. A population study was not done. [Tigyi and Shih-Fang (1962) previously reported a decline in resting potential and excitability in solutions made about 4T with sucrose added to Ringer]. These data cast some doubt on the measured tetanic tensions at 3T but do not affect the conclusions reached in this paper. In all cases, both elevated $[\text{K}^+]_o$ and electrical stimulation were used to depolarize the membrane and produce contraction.

Caffeine Contractures and Hypertonic Solutions

The decline of tension with increasing tonicity described above contrasts with the report of Caputo (1966) that caffeine contractures are potentiated by hypertonic solutions (1.9T) in single fibers or small single layered bundles of

muscle fibers. This apparent discrepancy might be due to the different mode of action of caffeine; it stimulates chemically, releasing Ca^{++} from intracellular stores and bypassing the normal depolarization pathway. In order to investigate this possibility we studied the effects of a range of caffeine concentrations on the caffeine contractures of muscles in normal and hypertonic solutions. Caffeine produces contractures that are somewhat variable in time course and in magnitude and has variable deleterious effects on the subsequent muscle performance. Many muscles were used for only a single caffeine contracture. Results were discarded if the muscle did not relax to less than 5% of peak contracture tension following washout of caffeine. Also, experiments were terminated when tetanic tension fell to one-half of the initial tetanic tension. Even so, there was much variability in the data on these "reversible" caffeine contractures.

Fig. 3 shows a plot of the average peak caffeine contracture tension as a function of caffeine concentration for 1T, 2T, and 3T solutions. The peak caffeine contracture tension is expressed as a fraction of the 1T tetanic tension. In the range of caffeine concentrations used by Caputo (1966), near 0.5–3 mM, we also find that hypertonic solutions potentiate caffeine contractures but not by as much as indicated by Caputo. The hypertonicity used by him, 1.9T, is close to our 2T value. However, at higher caffeine concentrations where the maximum caffeine contracture tension occurs, hypertonic solutions decrease the maximum tension. The maximum reversible caffeine contracture tensions are plotted in Fig. 1 at 1T, 2T, and 3T. It can be seen that only at 3T is there a decline of tetanic tension below the maximum caffeine contracture tension. In addition, caffeine still produced contractures of about 10% of the tetanic

FIGURE 3. Peak caffeine contracture tension as a function of $[\text{caffeine}]_0$ for 1T, 2T, and 3T solutions. The average peak caffeine contracture tension as a fraction of the 1T tetanic tension is the ordinate. The $[\text{caffeine}]_0$ in mM is the abscissa. Data are plotted for three tonicities, 1T, 2T, and 3T. The key identifies the symbols and separate curves. The bars represent ± 1 SEM. The number at each point is the number of determinations included in each point.

tension in 3T solutions at a time when tetanic and K contracture tension had fallen to zero, but cells were still excitable. Thus, hypertonic solutions decrease the maximum tension produced by the muscle no matter how elicited.

Effects of Hypertonic Solutions on Mechanical Threshold

The production of tension by caffeine in 3T solutions at a time when tetanic tension has vanished and cells are excitable, suggests that one of the steps in excitation-contraction coupling is deranged by hypertonic solutions. One possibility is that the mechanical threshold (the membrane potential at which minimal contraction occurs) is changed in hypertonic solutions. Measurements of mechanical threshold, the relationship between K contracture tension and potential, were made in 2T and 1T solutions since tension declines with time in a 3T solution (Fig. 2). The basic 2T solution is listed in Table I. The extra tonicity is made up mostly with NaCl. Results were obtained using three different combinations of "impermeant" anions. Tetanic tension was monitored and the experiment terminated when the maximum potassium contracture tension or tetanic tension had declined to less than two-thirds of the initial value at that tonicity solution. Many muscles were usable for both 1T and 2T runs using that criterion. The peak tension in the contracture was measured and expressed as a percentage of the peak tension of the first contracture at maximum $[K^+]_o$ for that tonicity. The small decline in muscle performance was estimated by taking the ratio between the tetanic tension preceding the particular K contracture and the initial tetanic tension at that tonicity. The

FIGURE 4. K contracture tension as a function of $[K^+]_o$. The fraction of the tension developed in a K contracture relative to a K contracture with all Na^+ replaced by K^+ is plotted against $[K^+]_o$. Key identifies different symbols. Notice the almost complete overlap of 1T and 2T data points.

peak K contracture tensions were corrected using the inverse of this ratio. This correction was usually not large but significantly reduced the variability in the values at any given potassium concentration. Fig. 4 is a plot of average peak K contracture tension as a function of $[K^+]_o$.

The tension- $[K^+]_o$ relation of Fig. 4 can be converted into a tension-voltage relation if the membrane potential as a function of $[K^+]_o$ is known. This was measured on muscles in both 1T and 2T solutions and for the three anion combinations used. The results for all three solutions were similar and are summarized in Table II. The results for the K methylsulfate-Na isethionate solutions are plotted in Fig. 5. All the curves show a slope of 52–55 mv/decade

TABLE II
DATA FROM POTENTIAL vs. LOG $[K^+]_o$ PLOTS

In this table are collected the data from the plots of membrane potential vs. log $[K^+]_o$ like that illustrated in Fig. 5 for the three combinations of anions used. The values of α and $[K^+]_i$ are computed as indicated in the text. The separation of the curves indicates for the linear part of the curves how much more depolarized the membrane potential is in the 1T solutions compared to that in the 2T solutions for a given $[K^+]_o$.

Solution Impermeant anion	Tonicity	Separation of 1T and 2T curves		Computed $\alpha = P_{Na}/P_K$	Computed $[K^+]_i$
		Slope <i>mv/decade</i>	<i>mv</i>		<i>mM</i>
Propionate	1T	53	13	2×10^{-2}	120
Propionate	2T	52	13	2×10^{-2}	202
Methylsulfate	1T	52.5	13	0.9×10^{-2}	118
Methylsulfate	2T	52	13	2.0×10^{-2}	211
Methylsulfate-isethionate	1T	55	17	0.5×10^{-2}	108
Methylsulfate-isethionate	2T	55	17	1.1×10^{-2}	230

for the linear portion at higher $[K^+]_o$, a separation of 13–17 mv between the 1T and 2T curves, and a concave upward departure from linearity (on the semilogarithmic plot) at the lower values of $[K^+]_o$ in 2T.

P_{Na}/P_K (α) and $[K^+]_i$ were computed (see Table II) using the method of Woodbury (Woodbury et al., 1970). $[K^+]_i$ increases by a factor of about two as the tonicity is increased from 1T to 2T. Thus the assumption of a doubling in $[K^+]_o$ with the doubling of external tonicity is justified. For two of the three anion combinations, α was also increased by a factor of two. These calculations assume that chloride is distributed in equilibrium with the membrane potential which is nearly the case because of the constant $[K]_o[Cl]_o$ product used. Since α is not zero, the $(\alpha[Na] + [K])[Cl]$ product should be kept constant (Geduldig, 1968). However, the error is only a few per cent.

The relationship between K contracture tension and membrane potential

can be obtained from tension vs. $[K^+]_o$ and the potential vs. $[K^+]_o$ relations. Fig. 6 shows the tension-potential relationship for potentials near threshold obtained by combining data of Figs. 4 and 5 (or appropriate curves for the different anions). It can be seen that tension was produced at a smaller membrane depolarization for the 2T solutions than for the 1T solutions in all cases. In order to obtain the mechanical threshold a least square straight line was fitted to the data above the foot of the curve. The intercept of this line with the potential axis was taken as the mechanical threshold. Straight lines are drawn for the three different anion combinations used in the 2T experiments.

FIGURE 5. Membrane potential as a function of $\log [K^+]_o$ in 1T and 2T. The average measured membrane potential in millivolts is plotted against $\log_{10}[K^+]_o$ for $[K^+]_o$ expressed in mM. Potential data are from the Na-isethionate, K-methylsulfate experiments. The open squares are for the 1T solutions. The filled squares are for the 2T solutions. The derived data from this curve and the curves from the two other anion combinations are collected in Table II. The curves were fit by eye.

A single line is drawn for the 1T experiments because there was no significant difference between the lines for the different anions. These data on mechanical threshold are collected in Table III. As can be seen, there is a difference between the mechanical thresholds for the 1T and 2T solutions with the mechanical threshold being closer to the resting potential for all 2T solutions.

Effects of Hypertonic Solutions on the Membrane Potential-Repriming Relation

The above data on mechanical threshold shifts in hypertonic solutions make it unlikely that hypertonic solutions cause any uncoupling between excitation and contraction due to failure of depolarization to reach mechanical threshold. However, since hypertonic solutions shift the mechanical threshold towards

the resting level and also depolarize the membrane towards the threshold, there is a distinct possibility that the contractile mechanism is partially inactivated in the 3T solutions. A muscle which has undergone a depolarization contracture and relaxed cannot contract again until the contractile mechanism has been reprimed by a period of repolarization. Hodgkin and Horowicz

FIGURE 6. K contracture tension as a function of membrane potential for 1T and 2T. The average fraction of peak tension in a K contracture is plotted against the average membrane potential in millivolts for the $[K^+]_o$ and tonicity of the contracture solutions for both 1T and 2T solutions. The symbols are identical to those of Fig. 4, with open symbols and solid symbols for 1T and 2T, respectively. A single least square line is drawn for the 1T data points above those at the foot of the curve. Least square lines are drawn for each of the 2T curves with different anion combinations. The average resting potentials in 1T and 2T are -92 and -83 mv, respectively.

TABLE III
MECHANICAL THRESHOLDS

The data from Fig. 6 on the mechanical thresholds, intercepts of the least square lines on the potential axis, are collected for the 1T and 2T solutions and for the three separate combinations of anions used. The results from the 1T experiments are not significantly different and are pooled. The pooled 2T result is also listed for comparison. The differences between the 1T and 2T data are listed.

Solution	Mechanical threshold	Shift from 1T value
	mv	
1T combined	-51	
2T combined	-63	12
2T Na-K propionate	-65	14
2T Na-K methylsulfate	-62	11
2T Na-isethionate	-63	12
K-methylsulfate		

(1960 *a*) described this phenomenon and termed the recovery process re-priming or restoration. They found that the dependencies of contracture tension and restoration on membrane potential were almost mirror images of one another (see their Fig. 11), i.e. restoration is almost complete at the mechanical threshold and restoration is almost absent at depolarizations producing maximum contractures. In order for lack of restoration to be an explanation of the E-C uncoupling seen in hypertonic solutions, the relationship between restoration and membrane potential must be shifted toward the resting potential, in the same direction as the mechanical threshold changes observed above, and to the point where restoration is not complete at the resting potential.

Fig. 7 shows the relationship between the fraction of restoration of peak K contracture tension and the membrane potential (set by the $[K^+]_o$ of the recovery solution) for 1T and 2T solutions. This figure shows that the restoration shifts in the 2T solutions in the same way as the mechanical threshold so that the fractional restoration at mechanical threshold voltages is nearly the same in 1T and 2T solutions. The shift caused by the 2T solutions is somewhat irreversible as shown by the difference between the data taken in 1T before (open squares) and after (open circles) exposure to the 2T solutions. If only the initial exposures to the solutions are considered, the restoration curves and the mechanical threshold curves are mirror images in both 1T and 2T.

Effects of Divalent Cations on Tension in Hypertonic Solutions

Since divalent cations play such a prominent role in E-C coupling, their effect on the tension decrease produced by hypertonic solutions was investigated.

FIGURE 7. Restoration as a function of membrane potential. The fraction of restoration of the K contracture tension after recovering for 2 min in a solution of differing $[K^+]_o$ is plotted against the average membrane potential for that $[K^+]_o$ and tonicity. The open symbols stand for 1T, the solid symbols for 2T. The open squares are data taken during the initial exposure to 1T. The open circles include also data taken after an exposure to 2T. The mechanical threshold lines are drawn in for reference. For these lines the ordinate is the fraction of peak tension. The average resting potentials in 1T and 2T are -92 and -83 mv, respectively.

Lüttgau (1963) found that elevated $[Mg^{++}]_o$ and $[Ca^{++}]_o$ can prolong activation in a K contracture and shift the mechanical threshold in 1T solutions. Control K contractures after 10 min in a 3T solution were followed by 5–10 min of soaking in a 3T solution with 18 mM $[Ca^{++}]_o$ or $[Mg^{++}]_o$ added to the normal 1.8 mM $[Ca^{++}]_o$ and then test K contractures were produced. The presence or absence of elevated divalent cation concentration in the K contracture solutions had no effect on the results. In seven of the eight experiments using elevated calcium, the tension of the second K contracture was elevated over the initial contracture; elevated $[Ca^{++}]_o$ restored the K contracture tensions from an average of 6.7 to 12.9% of the maximum tetanic tension in the 1T solution.

The effects of elevated $[Mg^{++}]_o$ were variable. The test contracture was higher than that of the control in four cases, the same ($\pm 20\%$) in five cases, and lower in three cases. K contracture tension after Mg treatment averaged 5.4% of the 1T tetanic value compared to 5.0% before treatment. However, since the tension normally declines steadily in the 3T solutions this lack of change could represent a potentiation.

Since divalent cations can affect the properties of both resting and excitable membranes (Frankenhaeuser and Hodgkin, 1957), they could possibly restore E-C coupling in an indirect manner through the membrane potential as well as more directly by a shift in mechanical threshold. It was found that high $[Ca^{++}]_o$ 3T solutions produced hyperpolarizations of only about 3 mv while the high $[Mg^{++}]_o$ 3T solutions produced no hyperpolarization. Ca^{++} has a greater influence on both tension and potential than Mg^{++} as has been found by others (Frankenhaeuser and Hodgkin, 1957; Lüttgau, 1963). Thus the possibility is left open that some component of restoration in high $[Ca^{++}]_o$ may be due to this small hyperpolarization.

Attempts to Repolarize the Muscle Fibers in Hypertonic Solution

The shifts in the mechanical threshold and the repriming curves with tonicity and the restoration of tension in 3T solutions with elevated $[Ca^{++}]_o$, are both consistent with the hypothesis that depolarization produced by 3T reduces contraction due to lack of repriming. Thus an attempt was made to repolarize muscles in 3T solutions by changing the bathing medium. A number of different 3T solution changes were tried: (a) replacing all the Na^+ with either choline⁺ or Tris⁺, cations that may be less permeable than Na^+ , (b) decreasing the $[K^+]_o$ in the presence of Na^+ , choline⁺, or Tris⁺, (c) bathing the muscle fiber in a low $[Cl^-]_o$ 3T solution for 15–30 min to try to deplete $[Cl^-]_i$, then returning the muscle to a solution with normal $[Cl^-]_o$, and (d) the same as (c) except for replacing Na^+ with Tris⁺ and using normal or half-normal $[K^+]_o$. In none of these cases was either repolarization or restoration of K contracture tension observed. A small repolarization and restoration of K contracture

were seen in elevated $[Ca^{++}]_o$. However, because of the many effects of Ca^{++} on the E-C coupling mechanism (Lüttgau, 1963) and the minimal repolarization (3 mv), the restoration of K contracture tension cannot be attributed to repolarization alone.

Effect of Tonicity on Muscle "Resting" Tension

D. K. Hill (1968) found an increase in resting tension of frog sartorius muscle as the tonicity of the bathing solution was increased, which increase was sustained as the solution was made hyperosmotic with impermeant substances. We also observed an increase in the resting tension of the muscles bathed in solutions made hypertonic with sucrose or sodium chloride but, in contrast to Hill's observations, the major component of this change was transient, taking about 30 sec to reach peak value and decaying to half of the maximum value in another 30 sec. A small, more sustained component was found but this was less than 10% of the total change in resting tension. Peak values of tension changes averaged 3.3 and 21.6% of the 1T tetanic tension going from 1T to 2T and from 1T to 3T solutions, respectively. These values are much larger than those of Hill (1968). The time course of the onset of the resting tension change was qualitatively the same as the changes in muscle volume as observed by measuring the diameter of the whole toe muscle. However, the tension declined even though the muscle volume remained decreased.

The marked difference in the responses to hypertonic solutions of whole sartorius muscles and small bundles of toe or semitendinosus muscle fibers may be due to differences in diffusion time. If the phenomenon in a single fiber is mainly a phasic increase in tension with a small tonic increase (regardless of the mechanism), then the long diffusion equilibrium time in a sartorius might give rise to a weak, sustained contraction. Insufficient data were obtained to test this possibility.

DISCUSSION

Hypertonic Solutions and Contractile Tension

The major effect of hypertonic solutions on frog fast skeletal muscle fibers is to decrease tension no matter what method of stimulation is used. Our results on the variation of tetanic tension with tonicity confirm and extend those of Howarth (1958) obtained on frog sartorius muscles. In addition, we measured twitch, K contracture, and caffeine contracture tension on smaller muscle bundles where diffusion and solution equilibration are more rapid.

Hypertonic solutions could act either to affect normal calcium release and interaction with filaments or to have a direct effect on contractile tension. Since it is generally believed that caffeine releases calcium directly from internal stores, probably from the sarcoplasmic reticulum (Weber and Herz,

1968; Sandow, 1965), the comparison of maximum reversible caffeine contracture tension and tetanic tension helps to differentiate between the two possibilities. The maximum reversible caffeine contracture tension decreases with increasing tonicity in the same manner as the tetanic tension (Fig. 1). If it is assumed that the maximum caffeine contracture tension at each tonicity is equal to the maximum tension the muscle can generate under maximal calcium stimulation, the caffeine data suggest that the major effect of hypertonic solutions is directly on the contractile tension rather than on an earlier step in E-C coupling.

The possible direct effects of the hypertonic solutions on contractile protein interactions have been previously discussed by others. Howarth (1958) attributed the effect of hypertonic solutions to an increased internal viscosity. Podolsky and Sugi (1967) showed that hypertonic solutions reduced contraction velocity in skinned fibers. In isolated protein systems, increased ionic strength decreases the actomyosin ATPase activity (Hasselbach, 1952; cf. Weber and Herz, 1963). Since the ATPase rate is closely related to the maximum shortening velocity (Bárány, 1967), these data are more applicable to shortening than to tension. However, our data and the data of April et al. (1968) on the effects of ionic strength and fiber volume on the tension produced by an injection of calcium into single crayfish muscle fibers are relevant to the tension per se. April et al. (1968) showed that the tension produced by injection of a fixed amount of calcium declined steeply as the tonicity of the external solution was increased. Their data and those of Caputo (1968) indicate that the variation of maximum tension with changes in the external solution is correlated more with changes of ionic strength inside the muscle fiber than with changes in fiber volume. However, these experiments cannot differentiate between the two possibilities that increased ionic strength (*a*) decreases the maximum tension produced by the contractile proteins or (*b*) increases the Ca^{++} requirement for a given amount of activation. The latter is certainly a possibility in the light of the data of Fuchs et al. (1969) which show that an increase in ionic strength provides an extra binding site for calcium on troponin with the same binding constant as the initial site. Nevertheless, the data strongly indicate that the major portion of the tension decline in hypertonic solutions is due to the direct effects on the contractile proteins of the increase in ionic strength.

In addition to a direct effect of ionic strength on contractile proteins discussed above, there may be indirect effects such as changes in filament lattice spacing in hypertonic solutions affecting contractile strength. Hypertonic solutions decrease the filament lattice volume (Rome, 1968). The data of Edman and Andersson (1968) indicate that for 20% variations in tonicity about the normal value, the "tension per bridge" is decreased by hypertonic solutions, increased by hypotonic solutions, and affected little by variations

in filament lattice spacing caused by changes in sarcomere length from 1.5 to 3.0 μ . On the other hand, Hill (1968) showed that the twitch tension decline in going from 1T to 2T solutions was greater at long muscle lengths than at short muscle lengths, indicating that filament separation may play some role in the tension decline.

The Disruption of E-C Coupling in 3T Solutions

Our data show that 3T solutions probably cause some disruption of the normal E-C coupling mechanism: (a) in 3T solutions tetanic and K contracture tensions decline to zero from a value near the average maximum caffeine contracture tension (10% of 1T tetanic tension) at this tonicity. At this time, caffeine contractures of 10% of 1T tetanic tension can be elicited; (b) once the K contracture tension has declined, elevated $[Ca^{++}]_o$, 19.8 mM, restores K contracture tension to 13% of 1T tetanic tension, a value not significantly different from the maximum caffeine contracture tension at this tonicity. This disruption is probably a true E-C uncoupling rather than just an inability to depolarize since cells are excitable when tetanic tension is zero and depolarization is achieved by elevating $[K^+]_o$ as well as by action potentials.

The decline of tension above that expected for the direct effect of elevated tonicity on contraction discussed above could imply that the myoplasmic $[Ca^{++}]_{free}$ has decreased over that normally produced by depolarization or that the calcium requirement for a given level of tension has increased. On any particular depolarization the former could be due to a decreased calcium release, i.e. an effect on true E-C coupling (Sandow, 1965), or to more effective calcium uptake by the sarcoplasmic reticulum. However, because of the cyclic turnover of the releasable calcium pool through release and uptake, it is difficult to differentiate between effects of tonicity on release or uptake. We have described the tension decline as an E-C uncoupling without wishing to imply that the primary effect of elevated tonicity is on calcium release mechanisms. We will discuss the tension decline in terms of calcium release, uptake, and requirement using the general term "E-C coupling" to signify all these processes.

The E-C uncoupling by 3T solutions is probably not due to a shift in the mechanical threshold such that the depolarization produced by action potentials or elevated $[K^+]_o$ solutions does not reach the mechanical threshold. Our data and those of E. Homsher (personal communication) demonstrate that the 2T solutions shift the mechanical threshold toward the resting level so that less depolarization is required than in 1T solutions. However, we have no data on the effects of 3T solutions on mechanical threshold (but see below).

There are a number of other hypotheses to explain the apparent excitation-contraction uncoupling.

1. Membrane depolarization in hypertonic solutions combined with a

shift in the mechanical threshold and the repriming curves toward the resting level produces a depolarization inactivation of the E-C coupling mechanism. This hypothesis cannot apply to muscles in 2T solutions in which the average resting potential is -83 mv compared to a potential of near -70 mv at which repriming is just complete. On the other hand, in 3T the resting potential declines to near -70 mv. If the shift with tonicity in the repriming curve is not reversed at 3T, restoration would not be complete at the resting potential. The restoration of the K contracture in high $[Ca^{++}]_o$ is consistent with this hypothesis in that increased $[Ca^{++}]_o$ shifts the mechanical threshold and repriming curves away from the resting level (Lüttgau, 1963). Since we were not able to test this hypothesis by repolarizing the muscle fiber membranes by a change of solution (except by a few millivolts in high $[Ca^{++}]_o$), this hypothesis is tenable but untested.

2. Another possibility is that since muscles are activated phasically during tetani and potassium contractures, a reduction in contraction velocity (Podolsky and Sugi, 1967; Howarth, 1958) in hypertonic solutions leads to a decrease in tension during activation. The partial return of the K contracture in high $[Ca^{++}]_o$ or $[Mg^{++}]_o$ is consistent with this hypothesis since Lüttgau (1963) showed that elevated $[Ca^{++}]_o$ prolongs activation in a K contracture. However, we were unable to show this prolongation in all cases at 1T. Also, in two experiments, stretching the muscle during activation in the 3T solution to extend the series elastic element did not produce extra tension above that due to stretch of the passive muscle. This negative result does not eliminate the hypothesis since the effect of stretch was not investigated thoroughly.

3. An additional possibility is that hypertonicity increases the rate of Ca sequestration by the sarcoplasmic reticulum (SR) so that although an apparently adequate amount of Ca is released on depolarization, an inadequate amount reaches the myofilaments. This possibility is unattractive since elevated KCl concentrations inhibit the ability of skeletal muscle microsomes (isolated SR) to (a) take up Ca in the presence of ATP (Martonosi and Feretos, 1964), (b) bind Ca in the absence of ATP (Carvalho, 1966), and (c) relax contracted glycerol-extracted muscle fibers (Takauji and Taniguchi, 1965). Since one of the effects of elevated tonicity is to increase $[K^+]_i$, the possibility that Ca is sequestered more effectively in hypertonic solution is unlikely unless isolated muscle microsomes have very different properties for Ca uptake than does intact SR.

4. A final possibility is that insufficient calcium is released by depolarization in 3T solutions to activate the contractile elements due either to an increased Ca^{++} requirement as suggested by the findings of Fuchs et al. (1969) or a decreased total quantity of releasable Ca^{++} . Experiments on Ca^{++} flux and total muscle Ca are needed to test this hypothesis.

Mechanical Threshold Shifts in Hypertonic Solutions

We found a threshold for contraction in 1T solutions of -51 mv (Table III); this is very near the -50 mv obtained by Hodgkin and Horowicz (1960 *a*) using single fibers and K contractures and the -48 mv mechanical threshold obtained by Costantin (1968) in local voltage-clamp experiments. In 2T solutions, the mechanical threshold shifted to about -63 mv (Table III).

The shift of mechanical threshold with tonicity is consistent with the hypothesis that the mechanical threshold is determined by the electric field in the membrane which depends on both the intracellular potential and the local surface potential due to fixed negative charges on the inside of the membrane. These negative charges would be partially neutralized by the elevated internal ionic strength. Surface charges have been postulated to explain similar effects; e.g., by Chandler et al. (1965) to explain shifts of the Na activation and inactivation curves produced by changes in internal ionic strength in perfused squid axons; by Hodgkin and Horowicz (1960 *b*) to explain the effects of nitrate and other anions on mechanical threshold in muscle fibers. In the former case, internal negative charges and in the latter case, external positive charges were postulated.

The density of fixed negative charge on the inside of the membrane necessary to produce the observed 12 mv shift can be estimated from the Gouy (1910) theory of the diffuse double layer (cf. Chandler et al., 1965). The result is $4 \mu\text{coul}/\text{cm}^2$ (less than twice that computed for squid axon by Chandler et al., 1965) assuming a capacity of $2 \mu\text{F}/\text{cm}^2$ of membrane (Falk and Fatt, 1964) and assuming a doubling of internal ionic strength from an initial value of 0.12. On this basis, the additional shift in mechanical threshold on going from 2T to 3T is calculated to be 5 mv. These calculations indicate that this is a quantitatively reasonable hypothesis.

Lorković (1967) found that the $[\text{K}^+]_o$ needed to produce a half-maximum K contracture increased with increasing external ionic strength and concluded that there are fixed negative charges on the outside of the membrane. External negative charges have also been postulated in squid giant axon by Frankenhaeuser and Hodgkin (1957) and in frog skeletal muscle fiber membranes by Lüttgau (1963), because of the effects of elevated $[\text{Ca}^{++}]_o$ on excitability parameters and on mechanical threshold, respectively. Negative charges on both the inside and outside of the membrane produce surface potentials whose effects tend to cancel when ionic strength, both inside and outside, is elevated simultaneously as with solutions made hypertonic with NaCl (as in our case). Thus this hypothesis cannot explain our results unless the density of net fixed negative charges (unneutralized negative minus unneutralized positive charges) is lower on the outside than on the inside. Lorković's data can be used to calculate surface charge density if one assumes a

$[K^+]_o$ -potential relation which is independent of external ionic strength. With the use of our 1T $[K^+]_o$ -potential data, the calculated external negative surface charge density is about $1 \mu\text{coul}/\text{cm}^2$, one-fourth of the internal negative charge density calculated above. This result is reasonable since Ca^{++} presumably binds to these fixed negative charges and since the ratio $[Ca^{++}]_o : [Ca^{++}]_i$ is very large, it would be expected that more outside than inside negative charges are neutralized by Ca^{++} .

The repriming curve and the mechanical threshold curve are both shifted by approximately the same amount when either tonicity or $[Ca^{++}]_o$ is increased (Lüttgau, 1963) indicating that both repriming and mechanical threshold may be related to membrane potential and surface potential in the same way.

Membrane Properties in Hypertonic Solutions

Hypertonic solutions affect the resting membrane properties of muscle cells by increasing the ratio of $P_{Na} : P_K$ by a factor of about two in 2T solutions. This is in the same direction as the change observed by Baker et al. (1964) with increased internal ionic strength in perfused squid giant axon. The failure of attempts to repolarize the cells in 3T by replacing various ions gives an indication of the relative ion permeabilities of the membrane. The fact that cells did not repolarize when Na^{++} was replaced with either choline⁺ or Tris⁺ indicates that the membranes in 3T solutions may be permeable to these ions. The lack of effects of changes in $[Cl^-]_o/[Cl^-]_i$ on membrane potential in 3T solutions indicates that the chloride ion permeability may be less dominant at 3T than at 1T. This suggestion is consistent with the data of Sperelakis and Schneider (1968) but it is opposite to what one would expect on the basis of neutralization of fixed negative charges. However, the dependence of chloride conductance on pH (Hutter and Warner, 1967) indicates that this simplified argument must be used with caution.

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