On the Inhibition of Muscle Contraction Caused by Exposure to Hypertonic Solutions

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ABSTRACT The evidence supporting a site of inhibition of excitation contraction (E-C) coupling near the plasma membrane (the "glycerol effect," the K⁺-potentiating effect) for muscle in hypertonic solution was reinvestigated. It was found, using whole frog sartorii, that there was a rehydration of muscle soaked in glycerol Ringer after 30 min and a large swelling (to 140% after 1 hr soaking) upon return of the muscle to normal Ringer, suggesting that significant amounts of glycerol enter the fibers during this time. While contrary to the original report of the glycerol effect, this finding was consistent with other studies involving the use of single fibers. Also reexamined was the potentiating effect of K⁺ on the hypertonic inhibition of muscle contraction. It was found that muscles exposed to this KCl pretreatment swell so that they are less dehydrated in hypertonic solutions, thus accounting for the observed potentiation. After being treated instead with a K₂-tartrate Ringer solution, muscles did not swell and, as determined with twitch recordings, did not display any potentiation in hypertonic solutions—even though the [K⁺] was higher than an osmotically equivalent KCl solution. The evidence was thus consistent with alternative hypotheses in which inhibition of contraction occurs at a later stage in E-C coupling or involves the contractile process itself.

INTRODUCTION

On exposure to hypertonic solutions muscles fail to contract as a result of either nerve (Thesleff, 1959) or direct stimulation (Overton, 1902). The failure of nerve stimulation is well understood—the osmotic changes cause a diminution in quantal release of acetylcholine (Hubbard et al., 1968). The failure of direct stimulation is not so well understood. It could be either a failure in the processes linking the depolarization of the muscle membrane to calcium release from the sarcoplasmic reticulum and the consequent activa-
tion of myosin adenosine triphosphatase (ATPase), which will be termed “excitation-contraction” (E-C) coupling, or the failure of actin and myosin to move normally, which will be termed a “failure of contractility.”

It is clear that the effects of hypertonic solutions upon muscle contraction are a function of the degree of hypertonicity. Gordon and Godt (1970) have shown that both contractility and, to a minor extent, E-C coupling are impaired when muscles are exposed to solutions with three times the normal tonicity. In the range of twice the normal tonicity the balance of the evidence indicates that, as suggested by Howarth (1958), inhibition of contraction is due to a failure of contractility. Of major importance is the report that the heat of activation is measurable in such preparations after direct muscle stimulation (Hill, 1958), which implies that there is no failure of E-C coupling. A complementary experiment is the finding that contractures caused by caffeine, which mobilizes calcium directly, are depressed by soaking preparations in hypertonic solutions (Podolsky and Sugi, 1967; Gordon and Godt, 1970), as is the direct effect of injected calcium upon skinned muscle fibers previously exposed to hypertonic solutions (April et al., 1968).

Only two groups of experiments stand in the way of complete acceptance of failure of contractility as the major cause of inhibition of contraction. Firstly, Fujino et al. (1961) and Yamaguchi et al. (1962) report that whole frog sartorius muscles, paralyzed by soaking in hypertonic glycerol (for up to an hour), recover the ability to contract while remaining dehydrated for the whole period. Secondly, Fujino and Fujino (1964) report that presoaking sartorius muscles in isosmotic potassium solutions will prevent inhibition of contraction by hypertonic saline, although no appreciable change in dehydration as a result of the presoaking was reported. Both experiments were interpreted by their authors as indicating a flaw in E-C coupling in inhibited muscles.

In the course of experiments upon neuromuscular transmission in frog sartorii previously soaked for an hour in glycerol Ringer, we became curious as to the effects of this presoaking and repeated the experiments of Fujino et al. (1961), Yamaguchi et al. (1962), and Fujino and Fujino (1964) with certain modifications. Our results indicate that these experiments do not support these authors’ conclusions. A preliminary report of some of our experiments has been published (Miyamoto and Hubbard, 1970).

**METHODS**

Matched pairs of sartorii from *Rana pipiens* were used for all experiments. The animals were kept in a cage with a continuous cold water (18°C) flow, although all investigations were conducted at room temperature (22°C–25°C). In experiments in which muscles were weighed before, during, and after exposure to hypertonic solutions, excess moisture was removed from the muscle before weighing by blotting it between
two pieces of filter paper (Bozler, 1959; Yamaguchi et al., 1962). Muscles were then weighed on a Sartorius 2400 analytical balance (sold by Brinkman Instruments, Inc., Westbury, N. Y.). Since the complete cycle of blotting, weighing, and returning the muscle to solution took no longer than 1.5 min, an interval of 2 min was used between weighings of the test muscle and the control.

Plots of muscle weights were made by first converting each value to a percentage of the weight at time zero. Regression lines were then drawn through the points for the muscle in control Ringer and the values for the test muscle were corrected accordingly. The results from several experiments were then averaged (Figs. 2 and 3).

The control Ringer solution was a modification of Adrian's (1956) to which 1 g/liter glucose was added and 8 mM NaCl subtracted with a resulting osmolarity of 220 mosmol. The final composition was (in millimoles per liter): NaCl 107, KCl 2.5, CaCl$_2$ 1.8, NaH$_2$PO$_4$ 0.85, Na$_2$HPO$_4$ 2.15, and glucose 5.56. A KCl-Ringer solution was made by simply replacing the NaCl with KCl with no consequent change in osmotic pressure. However, in order to make an equivalent isosmotic K$_2$-tartrate Ringer, it was necessary to replace the NaCl with only 85 mM K$_2$-tartrate, since there are two cations for every anion. The concentration of K$_2$-tartrate in solution required to maintain isosmolarity was estimated as follows. The degree of dissociation ($\alpha$) of the salt was found for 80 and 90 mM solutions by comparing the measured osmolarities (using freezing point depression osmometry, Precision Systems Inc., Framingham, Mass.) with the theoretical osmolarities, assuming complete ion dissociation. The results were 198/240 mosmol = 0.825 and 220/270 mosmol = 0.780 for the respective concentrations. By interpolating between these two points, it was estimated that an 85 mM solution would have an $\alpha$ of 0.80. It was further assumed that the remaining salts in normal Ringer, exclusive of NaCl, were on the average also 80% dissociated. Thus 85 mM X three ions = 255 mosmol + 24 mosmol (contribution of other salts) = 279 mosmol X 0.80 = 223 mosmol. Measurement of the actual solution showed an osmotic pressure of 222 mosmol, well within the accuracy needed. Thus the $\alpha$ for the divalent K$_2$-tartrate salt at that concentration was about 0.80, as opposed to about 0.95 for a similar univalent KCl solution. Osmotic pressures of all solutions were routinely measured using the above technique.

Muscle twitch tensions (Fig. 4) were recorded isometrically using a Grass FT. 03 C force-displacement transducer (Grass Instrument Co., Quincy, Mass.) coupled to a Beckman Type R "Dynograph" recorder (Beckman Instruments, Inc., Fullerton, Calif.) with suitable amplification. For these experiments the frog patella and part of the pelvis were also removed, so that the ends of the muscle could be secured with surgical clips. The muscle was fixed at one end to the bottom of the solution chamber (which contained 25 ml of solution) and stretched diagonally about 20° from horizontal, so that the opposite end could be attached to the transducer while still leaving the muscle submerged. The preparation was stimulated directly with platinum electrodes 1 cm apart (Howell, 1969), using supramaximal square pulses from a Grass S-8 stimulator and compatible isolation units (SIU 5). Changing of solutions and stimulation in air were carried out by emptying the chamber (<5 sec) using Pasteur pipettes coupled to a vacuum suction system.
Preliminary Observations on Osmotic Behavior of Sartorius Muscle

It has been postulated that muscle fibers behave like perfect osmometers (Krolenko and Adamyan, 1967; Birks and Davey, 1969) but there are in fact mechanical limitations at either extreme. In preliminary experiments the nature of the limitation at the dehydrated extreme and the relationship of muscle weight to osmolarity was established. Although this in itself is not new (see, e.g., Dydyńska and Wilkie, 1963), a good relationship has not been shown presumably because previous investigators have used calculated rather than measured estimates of the osmolarities of their solutions and have not used a control to detect the amount of regression in weight with time.

In these experiments Adrian's (1956) solution was made hypertonic with the addition of nonpenetrating sucrose or hypotonic by the reduction of NaCl and glucose and the exact osmolarities of the bathing solution were determined. The procedure was as follows. While the test muscle was transferred from solution to solution, the weight of the paired control muscle was recorded with time. An equilibration time of 20 min was allowed for the muscle in each solution before each value was recorded. Each point was then corrected for regression with time using the control as a guide (Fig. 1b). The actual sequence of solutions is shown in the order of the numbers in Fig. 1a.

As can be seen from Fig. 1a, the corrected points fall on a reasonably good curve in that points 1 and 8 (both normal Ringer) coincide well even though point 8 represents muscle which has been exposed to 700 mosmol Ringer. What this reflects then is the reversibility of the osmotic properties of muscle in solutions with osmolarities up to about 2½ times normal Ringer. It can also be seen that there is a plateauing of the muscle weight in solutions of about 520 mosmol, so that it is impossible to know the state of dehydration for muscles in solutions more hypertonic than this.

The fall in weight of control muscles (Fig. 1b) suggested either fiber damage or hyperosmolarity of the Adrian (1956) solution. Fiber damage due to the blotting procedure (see also the precautions of Dydyńska and Wilkie, 1963) was not a factor since the experiments of Adrian (1956) done without blotting showed the same type of regression. Adrian's (1956) solution has an osmotic pressure of 225 mosmol, whereas the extracellular fluid of our frogs had an osmolarity of 220 ± 4 mosmol. This figure represents the mean ± standard error of the mean, as do all data presented (Figs. 2–4). In these experiments fluid from two to three frogs was pooled for four separate determinations. The small difference between the solution and the fluid tonicity could presumably account for the fall in weight. Our control solution was adjusted to a tonicity of 220 mosmol by reducing its NaCl concentration to 107 m, a step which was further justified by the fact that Adrian's solution has a higher NaCl concentration than frog plasma (Fenn, 1936). This change reduced but did not abolish the fall in weight with time. For instance, in those experiments in which muscle weight was monitored for 140–200 min (Fig. 2), there was still a loss in weight of 2.34 ± 0.43%/hr (number of experiments, n = 8). A loss of some osmotically active substance with time (Dydyńska and Wilkie, 1963) seemed the most probable ex-
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**FIGURE 1.** Effect of osmotic pressure of Ringer on muscle weight. (a) Result of immersing a frog sartorius muscle in solutions with differing osmolarities as measured on a freezing point depression osmometer. The sequence of immersion is indicated by the numbers and also by the directions of the arrows. Points have been corrected for regression with time (see text) as shown in (b). (b) Regression of muscle weight with time, as recorded from the paired control muscle.

**FIGURE 2.** Weight changes of frog muscle in glycerol Ringer. Ordinate: muscle weight in terms of per cent initial weight. Abscissa: time in minutes. Points in this and subsequent figures show mean ± se and are corrected for regression (see Methods). The effect of soaking muscle in 420 mM glycerol Ringer for (A) 60 min, then returning to normal (open circles), n = 4, is shown. Points between 30 and 90 min are the means of eight preparations. (B) Soaking in glycerol Ringer for 115 min. Note the larger net overshoot for longer duration of soaking (n = 3). Note also the rehydration at 30 min and the extrapolation to 100%.
RESULTS

Exposure to Hypertonic Glycerol Ringer

Muscle weights initially decreased to about 80% of the control (Fig. 2) upon exposure to hypertonic glycerol, as reported by Yamaguchi et al. (1962). As Fig. 2 shows, however, muscle weights later increased again, as would be expected from the known ability of glycerol to enter frog sartorius muscle fibers (Bozler, 1961a; Howell, 1969). After only 30 min in glycerol Ringer there was a small but noticeable increase in muscle weight, i.e. the dehydration was not maintained during this time (Fig. 2, open circles between 30 and 90 min), as claimed by Yamaguchi et al. (1962). When the muscle was soaked for longer periods (115 min, Fig. 2 B, closed circles) the increase in weight was much more noticeable and displayed a linear relationship with time. Furthermore, extrapolation of this line (Fig. 2, heavy broken line) to the initial value (light broken line) suggested an equilibrium time of 160 min, corresponding well with the 150 min equilibration time found by Bozler (1961a) in the same preparation using isotopically labeled glycerol.

Upon returning the preparation to a normal Ringer solution there was a rapid and sizable gain in weight to a value significantly higher than the initial weight and dependent on the length of soaking in glycerol Ringer. Although Fujino et al. (1961) and Yamaguchi et al. (1962) reported that it was unlikely that glycerol entered muscle fibers during this short exposure to glycerol, both of the above observations are nevertheless consistent with the idea that glycerol does in fact penetrate into the fibers, a conclusion substantiated by Bozler's (1961a) experiments.

It should be noted that the weight changes shown in Fig. 2 are not due to the different densities of normal versus glycerol Ringer, for actual measurements of the two solutions revealed a discrepancy of only 9 g/liter of solution, or about 0.9%. That these differences are small is further indicated in experiments in which sucrose Ringer was used to maintain hypertonicity (Dydyńska and Wilkie, 1963). The weight increase there, due to density differences for 8.5 X normal Ringer, or almost 2000 mosmol, was estimated to be only 7–8%. Clearly, for our experiments involving the lighter molecule glycerol and only three-times the normal tonicities, the density factor is negligible.

Changes in the extracellular compartment, furthermore, exclusive of the transverse tubular-sarcoplasmic reticular (TT-SR) network, do not contribute significantly to the weight changes seen in whole muscle (Fig. 2), since the minimal (78%) and maximal (140%) weight values in Fig. 2 A concur...
with the respective values of 73 and 130% for single fibers soaked in 220 mM glycerol Ringer (Table I of Krolenko and Adamyan, 1967). Dydyńska and Wilkie (1963), using sucrose space measurements on whole muscle, have shown that there is no change in total extracellular space in up to two times the normal Ringer concentration and only about 5% increase in three times normal, while Birks and Davey (1969) estimate the changes in reticular volume to range from 11.5 to 18.5% of normal cell volume (with 13% normal) for osmolarities of 0.75–3.5 times normal. The agreement between the former “total” and the latter “reticular” extracellular space increases (5%) suggests that only the reticular extracellular space undergoes volume increases in hypertonic solutions. Finally, irrespective of the amount of contribution of these changes to measured muscle weights, it has been shown that volume changes in the TT-SR system occur in the direction opposite to changes in the fiber itself (Birks and Davey, 1969). Thus the weight changes seen in Fig. 2 are effectively less than the actual changes in fiber volume, making it impossible for the observed phenomena to be due to variations in the extracellular compartment.

The K⁺-Potentiating Effect

Fujino and Fujino (1964) reported that by presoaking muscle in “isotonic” KCl Ringer (in which NaCl is replaced by KCl) for periods of up to 30 min, the inhibition of contraction normally expected upon immersion in double-NaCl Ringer (Ringer made hypertonic with a doubled NaCl concentration) was prevented, and that contractions could still be elicited from muscles so treated. This effect was thought not to involve osmotic factors since the rates of dehydration as indicated by changes in fiber diameter for both presoaked and unsoaked muscles were approximately the same.

As Fig. 3 indicates, we repeated this procedure, monitoring, however, changes in weight with time rather than with muscle diameter. Also, in order to accentuate the effect of the KCl Ringer, the muscles were presoaked for 40 min rather than 30 min in KCl Ringer and left for 90 min rather than 60 min in the double-NaCl Ringer. Muscles gained weight in KCl Ringer. Upon immersion in the double NaCl Ringer, the test muscles lost weight at the same rate as the controls, but because of the presoaking, the absolute weights were nonetheless quite different. After 10 min, presoaked muscles were at their normal weight while the control muscles had fallen to 85% of their initial weight. After 50 min the presoaked muscle was at 83% of its initial weight while the control was maximally dehydrated (see Fig.1 a) at 73% of normal weight. Finally, upon return to normal Ringer, the presoaked muscle attained its initial value whereas the control muscle did not in the time allowed.
Our results show that the presoaked preparations were not as dehydrated as the nonpresoaked. The prevention of inhibition of contraction, then, could be due to the smaller amount of dehydration of the KCl-treated preparations rather than to any specific effect of $K^+$. The gain in weight displayed above by muscles soaked in KCl Ringer was postulated to be due largely to the entry of the anion of the K salt ($\text{Cl}^-$) as a result of the large concentration gradient and relatively high ion permeability. This hypothesis could be distinguished from that of Fujino and Fujino (1964) if a K salt could be found, the anion of which did not penetrate muscle cells. Under these circumstances, if Fujino and Fujino (1964) were correct, pretreatment with this salt should still potentiate contraction in double NaCl as did KCl. If, however, some swelling were required for the protective effect, no improvement should be seen. We chose the tartrate anion because of its large physical size (> glycerol), which makes it almost totally impermeable, and the physiological pH of its K salt (7.4). The use of the acetate and/or succinate salt was contraindicated by the possibility of these anions acting as metabolic substrates and thus being actively transported into the muscle fibers. Tartrate, on the other hand, does not occur naturally and has no known metabolic role.

The permeability to the tartrate anion was tested by following muscle weights in control Ringer for periods of from 30 to 60 min and then after immersion in isosmotic $K_2$-tartrate Ringer for at least 40 min. Although there was the expected K contracture on changing solutions, there was no change in weight in any of four sartorii examined.
To test the hypothesis that KCl potentiation was due to the lesser degree of muscle dehydration (Fig. 3), muscle twitch tension recordings were carried out. Muscles were either presoaked for 20 min with isosmotic KCl or K$_2$-tartrate or left untreated. Each measurement consisted of the average of four twitches, 10 sec apart. The results, although tending to vary from one muscle to the next, gave average responses as shown in Fig. 4. We never found any recovery of twitch to control levels, even if muscles were presoaked for up to 30 min in KCl, although KCl-presoaked muscles did give slightly larger twitch tensions (10%) than did muscles presoaked in K$_2$-tartrate. Furthermore, after 20 min the twitches given by the untreated control muscle and the K$_2$-tartrate–treated muscle were identical while the twitch tension of KCl-presoaked muscle was still somewhat higher. It should be noted also that in all these experiments twitch tensions returned to 80–90% of the presoaked control level within 10–15 min of return to normal Ringer, eliminating the possibility that the reduction of the twitch was due to muscle damage. We concluded then that the effects of KCl were due to the muscle swelling and the lesser dehydration in hypertonic solutions which followed, rather than to any specific effect of K$^+$.
DISCUSSION

Our results (Figs. 2–4) indicate that the evidence purporting to indicate a deficit in E-C coupling during inhibition of muscle contraction by hyperosmotic solutions can be otherwise interpreted. The finding (Fig. 2) that the sartorius muscle exposed to glycerol gains weight after an initial dehydration has also been reported by Krolenko and Adamyam (1967) and by Caputo (1968) using single fibers of frog muscles and short (5–10 min) exposures and seems consistent with the known permeability of the muscle membrane to glycerol (Bozler, 1961a). The difference between the results on single fibers and the present findings is undoubtedly due to the diffusion time required for the glycerol solution to equilibrate throughout the whole muscle (Krolenko and Adamyam, 1967). While Caputo (1968) has suggested that the failure of Yamaguchi et al. (1962) to observe a weight recovery in glycerol-soaked muscle was due to too short an exposure time, the present results show that by correcting for temporal regression, the recovery is indeed observable. We cannot however, explain the failure of Yamaguchi et al. (1962) to notice the rapid and sizable increase in weight (swelling) on return of the soaked muscle to normal Ringer (Fig. 2 A, B).

We view the return of contractility in glycerol-dehydrated muscle (the glycerol effect) as being due to two factors; the first is that there is in fact a rehydration at the time contractility returns. This increase in weight alone however, may be too small to explain the relatively large increase in contractions indicated by Fujino et al. (1961). Thus, a second factor is proposed, namely, a reduction in internal ionic strength. It is known that muscles soaked in glycerol or urea lose Na and K (Bozler, 1961b) due to the entry of the permeating solutes. This is not due to dehydration per se since a soaking with nonpermeating sucrose does not induce a loss of cations. This loss would explain the fact that the muscle remains relatively dehydrated (for the period 30–60 min), even though a substantial amount of glycerol, as indicated by the large swelling on return to normal Ringer, enters the fibers. The exchange of the nonelectrolyte glycerol (or urea) for cations therefore has the net effect of maintaining internal osmotic pressure while at the same time lowering the ionic strength. This is consistent then with the hypothesis that it is the increased ionic strength and not the dehydration in itself that inhibits contraction (Gordon and Godt, 1970).

The discrepancy between our results and those of Fujino and Fujino (1964) in the case of the KCl pretreatment (Fig. 3) presumably arises because they looked at muscle diameters only. According to extensive investigations by Blinks (1965), measurements of fiber diameter are subjected to gross random error and bias such that the examination of muscle weights, while
admittedly crude, may give a better indication of hydration states. In any case
Fujino and Fujino (1964) did report a small swelling in isosmotic KCl
(+4.5% of fiber width) and simple calculations assuming a circular cross-
sectional area and no change in length show that this change is equal to a
volume change of about +16%, comparable to our results (Fig. 3). A further
argument against the idea that K⁺ itself is responsible for the potentiation
observed is that one would expect an increased effect with higher K⁺ con-
centrations. Since each mole of K₂-tartrate gives rise to two equivalents of
K⁺, the isosmotic K₂-tartrate Ringer contained roughly 140 mM free K as
opposed to about 100 mM for a similar KCl Ringer, yet no twitch potentiation
was observed.

Swelling of muscles in "isosonic" KCl is a well known phenomenon, caused
by entry of both K⁺ and Cl⁻ into muscle fibers, for the internal [K⁺] remains
unchanged although fibers swell (Boyle and Conway, 1941; Adrian, 1956). We
would interpret the lesser dehydration of muscles presoaked in KCl when
immersed in twice normal NaCl, when compared with muscles not pre-
soaked, to the greater internal ionic concentration, especially of Cl⁻, achieved
by preloading. When in the double-NaCl Ringer, presoaked muscles are
faced with a large Cl⁻ concentration (214 mM) externally and very little Cl⁻
would be lost through diffusion fluxes. On return to normal Ringer, pre-
soaked muscle would still contain a high internal [Cl⁻] and would thus be
able to recover its initial weight. Unpresoaked muscle on the other hand,
having lost K⁺ due to dehydration and containing a low internal [Cl⁻]
initially, does not regain its original weight, and only after K⁺ is again
pumped in does it attain its normal weight.

Our results thus indicate no specificity of K⁺ per se in the above process,
for presoaking muscle in K₂-tartrate did not ameliorate the effects of twice-
normal NaCl (Fig. 4). In short, it appears that the recovery from inhibition
of muscle contraction in glycerol, and as a result of KCl pretreatment, can
both be explained by muscle rehydration and the consequent recovery of
muscle contractility, rather than by any major effect on E-C coupling.

This work was supported by National Science Foundation Grant GB-14294 to John I. Hubbard.

Received for publication 3 November 1971.

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