Inhibition by Hypertonic Solutions of Ca-Dependent Electrogenesis in Single Crab Muscle Fibers

GUILHERME SUAREZ-KURTZ and A. L. SORENSON

From the Department of Pharmacology, Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Dr. Suarez-Kurtz's present address is the Laboratory of Neurophysiology, College of Physicians and Surgeons, Columbia University, New York 10032, and Dr. Sorenson's present address is the Department of Physiology, Albert Einstein College of Medicine, Bronx, New York 10461.

ABSTRACT This study describes the effect of hypertonic solutions on isolated muscle fibers of *Callinectes danae*. Solutions of twice normal tonicity (2.0 T) inhibit both the normal graded membrane responses and the spikes induced by procaine, tetraethylammonium, or barium. The inhibition is maintained throughout exposure to hypertonic solutions prepared by addition of impermeant solutes such as NaCl, sucrose, or Tris-propionate, but is reversible on their withdrawal. In the presence of permeant solutes such as glycerol or acetamide, the inhibition is transient. In both cases the onset of inhibition of the depolarizing Ca electrogenesis is correlated with shrinkage of the fiber. In the case of permeant solutes, the time course of recovery of the graded responses or the spikes follows the recovery of the fiber volume. Changes in the passive electrical characteristics of the fibers due to hypertonic solutions were unrelated to the blockade of membrane Ca activation. The current-voltage relationship in hypertonic solution revealed no increase in depolarizing K activation. Inhibition of the graded membrane responses and spikes appears to be associated with depression of Ca conductance. Hypertonic solutions might affect the activation of Ca conductance through reduction of the electric field generated by fixed negative surface charges and/or morphological changes in the T tubules. Membrane depolarization elicited little or no tension in 2.0 T solutions while caffeine contractures (10 mM) with an amplitude of 76% of the maximal contractile ability could still be elicited. This indicates that direct effects of hypertonic solutions on the contractile apparatus were not responsible for loss of tension. The latter is attributed to the inhibition of the transmembrane Ca currents.

INTRODUCTION

The dissociation of mechanical from electrical events by hypertonic solutions in skeletal muscle was first described by Hodgkins and Horowicz (1957) who observed that single frog fibers in hypertonic media lost the ability to twitch, although action potentials of apparently normal kinetics could still be elicited. Two hypotheses for this effect of hypertonic solutions have been proposed: the first suggests that fiber shrinkage leads to increased intracellular ionic strength or viscosity which depresses the ability of the contractile proteins to develop

tension (Howarth, 1958; Podolsky and Sugi, 1967; Gordon and Godt, 1970); the second postulates that hypertonicity affects some step in the excitation-contraction coupling (ECC) process (Caputo, 1966; Gordon and Godt, 1970; Homsher et al., 1974).

In crustacean muscle there is evidence for both these mechanisms: April et al. (1968) showed that the amplitude of contractions elicited in crayfish fibers by intracellular Ca injections decreases as the intracellular ionic strength increases; Ashley and Ridgway (1970), using barnacle fibers injected with aequorin, demonstrated that the photoresponse due to Ca release into the sarcoplasm during membrane depolarization was decreased by hyperosmotic glycerol solutions.

We have investigated the effects of hypertonic solutions on the Ca-dependent electrogenic responses of the muscle fibers of the South American blue crab *Callinectes danae*. Our results indicate that hypertonic solutions depress this Ca electrogenesis and we suggest that at the tonicities used this is the predominant mechanism for the uncoupling of mechanical from electrical events in these fibers. A preliminary report has appeared previously (Suarez-Kurtz and Sorenson, 1974).

MATERIALS AND METHODS

The experiments were performed at room temperature (22–25°C) with isolated fibers from the extensor muscle in the meropodite of *C. danae*. The procedures followed in the isolation of single fibers, recording of membrane potential and isometric tension, and stimulation with intracellular microelectrodes have been previously described (Girardier et al., 1963; Reuben et al., 1967; Suarez-Kurtz, 1974). In these experiments point stimulation is used. Weak depolarizing stimuli elicit very small local tensions and no obvious electrogenesis. Larger stimuli give rise to graded membrane responses and contractions which are no longer localized to the tip of the current micropipette but spread across and along the fiber. These are the contractions recorded in our experiments; they do not occur in the absence of depolarizing electrogenesis. Inhibitory effects on the electrical properties are easily demonstrated since the electrical phenomena are recorded at a fixed point close to the current electrode and it is possible to increase the stimulus strength at that point to surpass the threshold level. Tension inhibition, on the other hand, could result from reduction in the spread of the active membrane responses due to changes in membrane properties such as decreased length constant (Table I). To allow for this possibility we also studied fibers in which the graded responses were converted by drug treatment into propagated spikes which spread the entire length of the fiber.

To determine the cable properties, current and voltage microelectrodes were inserted opposite each other, within 50 μm, in the middle of the fiber. The decay of potential with distance was obtained by moving the voltage electrode along the fiber without lateral displacement. Voltage deflection was plotted in the usual way (Fatt and Katz, 1953) to obtain the length constant (λ_m). The time constant (τ_m) was taken as the time required to reach 84% of the final voltage deflection at the original positioning (Hodgkin and Rushton, 1946). R_i, the specific internal resistivity, was obtained from λ_m and the effective membrane resistance (R_{eff}) at the initial electrode positioning from $R_i = \pi d^2 \cdot r_i$, where d is the fiber radius and $r_i = 2R_{eff}/\lambda_m$. The specific membrane resistance (R_m) was obtained from $R_m = 2\pi d \cdot r_m$, where $r_m = r_i \cdot \lambda^2$. The specific membrane capacitance was obtained from $C_m = \tau_m/R_m$.

The composition of the control saline was (mM/liter): NaCl, 360; KCl, 12.5; $CaCl_2$, 12.5; $MgCl_2$, 10; Tris-maleate buffer (pH 7.2), 5. The hypertonic solutions were prepared

by adding to the isotonic saline various amounts of Tris-propionate, Tris-Cl, NaCl, sucrose, acetamide, or glycerol. The tonicity (T) of all solutions was determined by freezing-point osmometry with the control saline used as reference (1.0 T, 750 mosmol/kg H_2O). In most experiments Tris-propionate was used to increase the tonicity of the bathing medium. Tris rather than Na was used to maintain the Na:Ca ratio constant, since this ratio is important in determining transmembrane Ca fluxes in crustacean muscle (Orentlicher and Ornstein, 1971). Propionate was used to avoid changes in the $K_0 \times Cl_0$ product. When the K concentration was increased, the Cl concentration was reduced to maintain the $K_0 \times Cl_0$ product constant. Cl-free hypertonic solutions were prepared by replacing Cl in the isotonic saline with either propionate or methane-sulfonate and by addition of various amounts of Tris-propionate.

Procaine, TEA, and Ba ions were used to convert the characteristic graded responses of these fibers into all-or-none spikes. Procaine·HCl (1–2 mg/ml) and $BaCl_2$ (2–5 mM) were added to the different solutions. When TEA (25–50 mM) was used, an osmotically equivalent amount of Na was removed.

RESULTS

Effects on the Graded Membrane Responses and Local Contractions

The influence of hypertonicity on the membrane and contractile responses of a single fiber to depolarizing current pulses is illustrated in Fig. 1. Hypertonic solutions (1.6 T and 2.1 T, prepared by addition of Tris-propionate) increased the depolarization threshold for, and changed the amplitude and time course of the graded membrane responses. The threshold for tension development also increased. The rate of relaxation of the induced contractions was reduced during exposure to the hypertonic media. The afterdepolarizations which followed the graded responses of these fibers (Suarez-Kurtz and Reuben, 1975) were prolonged by the hypertonic salines.

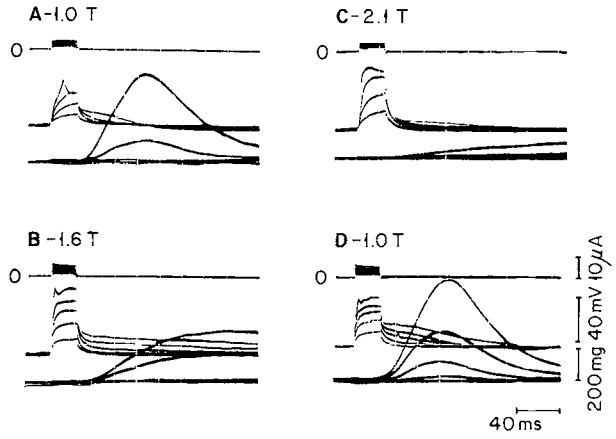

FIGURE 1. Effect of hypertonic saline on mechanical and electrical responses of an isolated muscle fiber. In all sections, the top lines are records of current, the middle tracings represent the membrane potential, and the bottom lines represent isometric tension. In this experiment and in those shown in Figs. 2–5 Tris-propionate was used to increase tonicity. Recordings were taken after 10 min of equilibration of the fiber with each solution. Note the reversibility of the effects of hypertonicity.

Blockade of graded electrogenesis was also observed when NaCl, Tris-Cl, or sucrose was used to increase the tonicity. A consistent finding with hypertonic sucrose solutions was membrane depolarization (6–10 mV) in contrast to the hyperpolarization observed with the other solutes (see below). With short exposures (5–10 min) to the hypertonic sucrose solutions the membrane repolarized on return of the fiber to the isotonic medium, and there was no sign of fiber damage.

In most cases the hypertonic saline replaced the normal saline in one step, as in the experiment shown in Fig. 1. In one series of experiments the tonicity was increased gradually, in steps of 0.05 T, to 1.8 T, and 2.5–5 min were allowed for equilibration in each solution. Here too, graded electrogenesis was inhibited.

Increasing the tonicity of the bathing solution with Tris-propionate up to 2.4 T had no immediate effect on the resting tension. However, 25% of the fibers soaked in solutions of 1.8–2.2 T for more than 30 min showed contractile waves. This activity was inhibited promptly when the fiber was replaced in isosmotic solution. The mechanism underlying these contractile waves was not further investigated in these crab fibers.

The inhibition of the graded membrane responses occurred within a few seconds of exposure to the hypertonic solutions and was maintained throughout the period of exposure (observed up to 2 h). The rate of recovery was also fast when the fiber was replaced in the control saline. These observations are illustrated in Fig. 2.

A fivefold increase in Ca_0 (by isosmotic replacement of Tris-propionate with Ca-propionate) did not reverse the inhibitory effects of hypertonicity on the graded responses.

Blockade of Ca Spikes and Twitches

Hypertonic solutions inhibited the action potentials induced by procaine, TEA, or Ba. Blockade of TEA- and procaine-induced spikes was observed when the tonicity of the medium was increased above 2.0 T. In both cases the spike electrogenesis recovered when the fibers were returned to the isotonic saline containing either agent. Spikes elicited in the presence of procaine in Cl-free propionate media were also inhibited by hypertonicity.

Fig. 3 shows the influence of hypertonicity of the Ba-induced spikes. At 2.2 T the generation of action potentials was blocked (Fig. 3 A). With progressive reduction of the tonicity, graded responses occurred (Fig. 3 B, C) before the recovery of action potentials. However, the graded responses at 1.9 and 1.6 T were not accompanied by local contractions as is observed at 1.0 T (see Fig. 1). Spikes with long-lasting plateaus at internal positive values of membrane potential were observed at 1.52 and 1.45 T; the amplitude of the accompanying twitches was 2.5 times larger at 1.45 T. Restoring the normal tonicity resulted in a shortening of the plateau of the action potential and a 10-fold increase in peak tension.

The evidence presented so far indicates that hypertonic solutions block membrane Ca activation which is responsible for both graded responses and spikes. The contractions associated with these membrane responses, i.e. local contractions and twitches, respectively, were also depressed. The inhibition of

the local contractions could be due in part to a decrease in the space constant of the fibers in hypertonic solutions (Table I); however, this cannot account for the loss of the twitches. One possibility is that the reduction in tension is

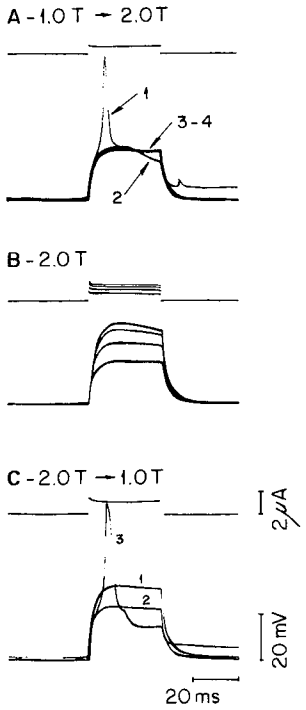

FIGURE 2. Electrical activity during onset and reversal of action of hypertonic solutions. In all panels the upper records are of current, the lower traces are the membrane potential. In the upper panel the fiber was stimulated every 6.3 s with depolarizing current pulses of equal amplitude and duration and the electrodes were kept in the fiber during the changes of solutions. The largest response was recorded 1 s before the hypertonic solution (2.0 T) was flushed through the chamber. A pulse applied 6.3 s later elicited a much inhibited response as indicated by its small amplitude and slow kinetics; the successive pulses failed to induce activation of the graded electrogenesis. The middle panel shows membrane responses to outward current pulses of varying intensities after 10 min of soaking in 2.0 T; the inhibition of the graded activation is seen clearly. Upon returning the fiber to isotonic saline (panel C) a sequence of events just the opposite of that shown in panel A was observed; thus the response recorded immediately before reintroduction of the isotonic solution showed no sign of activation. The next pulse still did not elicit the graded response but revealed the fall in membrane input resistance as the fiber recovered from the effects of the hyperosmotic medium. The third pulse elicited a response which was comparable in amplitude and time course to the control shown in the upper panel.

secondary to the blockade of the Ca-dependent membrane responses, another is the action of hypertonic solutions directly on the contractile apparatus. This is considered in the following section.

Effects of Potassium- and Caffeine-Induced Contractions

Hypertonic solutions inhibit the contractions elicited by high K_0. This is illustrated in Fig. 4. Record A shows the repetitive twitching and phasic contracture observed when K_0 was increased to 50 mM at a constant $K_0 \times Cl_0$ product. The tension after the twitches is approximately half that of the peak twitch tension. In 2.0 T, for the same K_0, no tension was observed (Fig. 4 B); further increase of K_0 to 116 mM at 2.0 T induced a slow-rising tension with an

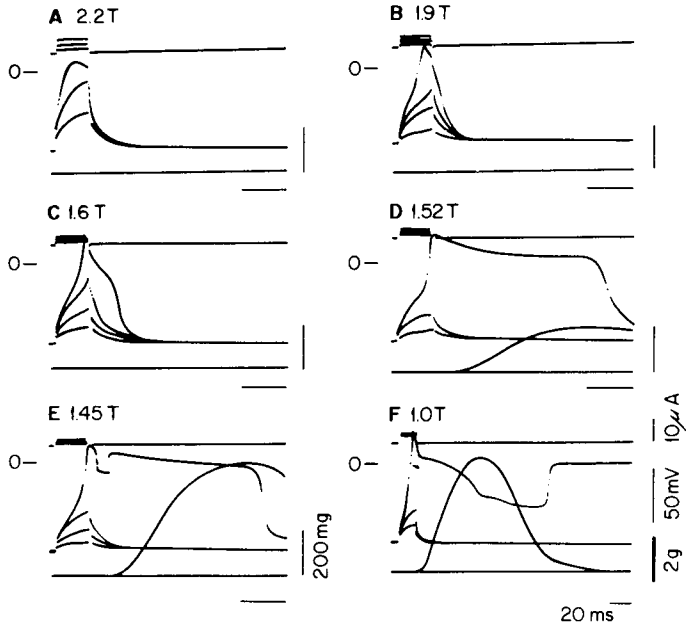

FIGURE 3. Blockage of Ba (10 mM) spikes by hypertonic saline. Each panel shows (from top to bottom): current, membrane potential, and tension. Note the absence of contraction in B and C in spite of electrical activation being present. Further description in the text.

amplitude of about 6% P_0 (Fig. 4 C). At 1.0 T, 116 mM K_0 elicits maximal contraction of these fibers. The inhibitory effect of hypertonic solutions on K-induced contractions does not result from a blockade of the K-induced depolarization of the fiber membrane (Fig. 4 E). Thus, in the range of 5–116 mM K_0, the relationship between membrane potential and K_0 shows a 48-mV change in membrane potential for a 10-fold change in K_0 in both 1.0 T and 2.0 T.

The block of the K contractures by hypertonic solutions (Fig. 4 B, C) may be influenced by a direct effect on the contractile proteins. To evaluate this possibility, we used caffeine contractures. Record D in Fig. 4 shows the contracture elicited by 10 mM caffeine in a solution made hypertonic to a value of 2.0 T. Peak tension was 1.5 kg/cm^2 and corresponded closely to the peak twitch tension observed in the same fiber (Fig. 4 A). In four experiments the average peak caffeine (10 mM) tension in 2.0 T was 1.82 kg/cm^2 (±0.13, SEM).

This is 76% of the maximum tension obtained in normal saline with elevated (116–261 mM) K_0. Peak tension obtained with 50 mM caffeine gave values similar to those obtained from K contractures. The smaller response to caffeine

TABLE I

EFFECT OF HYPERTONIC SALINE ON THE PASSIVE ELECTRICAL CHARACTERISTICS OF MUSCLE FIBERS OF *CALLINECTES DANAE*

Fiber	D	E_m	R_{eff}	τ_m	λ_m	r_i	R_i	r_m	R_m	C_m
						$\Omega \cdot mm^{-1}$				
	μm	mV	Ω ($\times 10^3$)	ms	mm	($\times 10^3$)	$\Omega \cdot cm$	$\Omega \cdot mm$	$\Omega \cdot cm^2$	$\mu F/cm^2$
Normal Saline										
1	94	62	23.6	1.6	0.443	10.7	74.2	2,100	62	25.8
2	165	64	11.1	1.6	0.734	3.03	64.8	1,632	84.6	18.9
3	184	65	11.2	3.2	1.15	1.95	51.8	2,579	149	21.5
4	169	60	13.3	1.63	0.790	3.37	75.6	2,103	112	14.6
5	195	60	15.2	3.50	0.781	3.89	116	2,372	145	24.1
6	161	60	10.4	1.25	0.672	3.10	63.1	1,400	70.8	17.7
7	189	64	20.5	1.75	0.625	6.56	184	2,563	152	11.5
8	183	63	24.0	2.5	0.830	5.78	152	3,982	229	10.9
9	192	70	16.7	3.2	0.984	3.39	98.1	3,282	198	16.2
Mean	170	63	16.2	2.25	0.779	4.64	97.7	2,446	134	17.9
SEM		1	1.8	0.29	0.068	0.89	15.0	266	19	1.7
Hypertonic (1.76 T) saline										
1	90	–	44.0	3.25	0.286	30.8	196	2,519	71.2	45.7
2	149	72	23.5	5.0	0.614	7.66	134	2,888	135	37.0
3	164	71	16.4	4.3	0.459	7.15	151	1,506	77.5	55.5
4	139	72	20.6	2.6	0.582	7.08	107	2,398	105	24.8
5	155	74	18.0	3.25	0.702	5.13	96.8	2,528	123	26.4
6	135	73	18.2	3.25	0.478	7.62	109	1,741	73.8	44.0
7	153	74	28.2	2.5	0.463	12.2	224	2,615	126	19.8
8	135	75	34.8	3.75	0.777	8.96	128	5,409	229	16.4
9	149	84	25.0	5.0	0.734	6.81	119	3,669	172	29.1
Mean	141	74	25.4	3.66	0.566	10.4	141	2,808	124	33.2
SEM		1	3.0	0.31	0.053	2.6	14	386	17	4.4

Length constants were obtained from least-squares fits of the potential *vs.* distance data with equal weight given to each point. The correlation coefficients for a linear regression averaged $R = 0.98$ for each set of data, with the lowest being $R = 0.95$. Saline was made hypertonic by the addition of Tris-Cl in all cases except fiber 8 where NaCl was used. The resting potential of fiber 1 in hypertonic solution was not recorded. The diameter referred to (D) is the cylindrical diameter, measured across the fiber at the point of insertion of the electrodes, in both the normal and the hypertonic saline. E_m is the resting potential, R_{eff} is the input resistance, τ_m is the membrane time constant taken to be the time to reach 84% of the final membrane voltage in response to a constant current step, λ_m is the membrane length constant, r_i is the internal resistivity for a unit length of fiber, R_i is the specific internal resistivity, R_m is the specific membrane resistance, and C_m is the specific membrane capacitance. SEM = standard error of the mean. $\times 10^3$ means that the number must be multiplied by 10^3 to obtain the proper value. In all experiments recorded here the hypertonic solution was made 1.76 times the normal tonicity.

in 2.0 T might be due to an effect on the contractile proteins or it might be due to the use of a smaller concentration of caffeine. The results do indicate, however, that the contractile apparatus is functional and that these fibers under our conditions are capable of developing tension in hypertonic solutions.

Effects of Hypertonicity on the Electrical Properties of the Resting Membrane

The effect of hypertonic solutions on the current-voltage (I-*E*) relationship is seen in Fig. 5. The input resistance (R_{eff}) of this fiber increased from 31 Ω to 36 Ω; increase in R_{eff} in hypertonic media was a common, although not invariable, finding in our study of passive electrical membrane properties (Table I). The

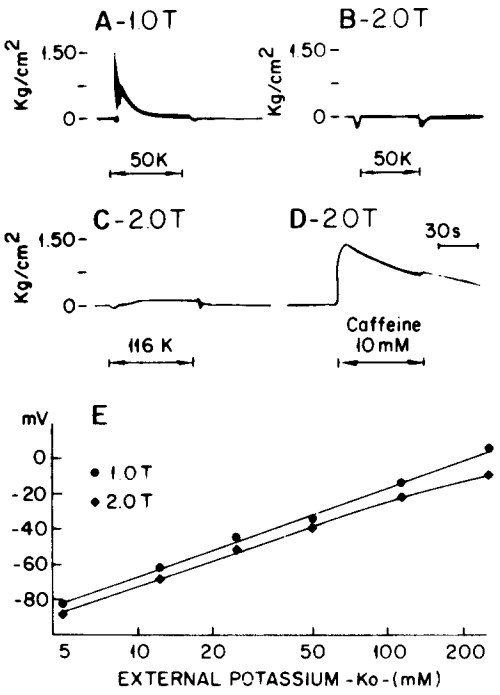

FIGURE 4. Effects of hypertonic solutions on K- and caffeine-induced contractions and on the relationship between K_0 and membrane potentials. Panels A–D show tension recordings from a single fiber exposed to increased K_0 or to caffeine; the challenges in B–D were made after an equilibration period of 10 min with 2.0 T hypertonic solution. E, the points in 1.0 T represent average values for from 3 to 14 fibers for each point. The points in 2.0 T were obtained from a single fiber exposed successively to 50, 5, 25, 116, and 261 mM K_0; between measurements in these solutions the fiber was returned to 2.0 T solution containing 12.5 mM K until the membrane potential returned to the control value.

data in Fig. 5 provide evidence against the possibility that inhibition of Ca-dependent electrogenesis by hypertonicity results from an increase in the outward K currents (see Discussion) since there is no sign of increased delayed rectification in the I-*E* plot.

The effects of hypertonic solutions (1.76 T) on the cable characteristics of crab muscle fibers are shown in Table I. Each fiber in this table served as its own control. Some experiments were also performed with tonicities up to 2.0 T with qualitatively similar results. In 1.76 T, the resting potential rose an average of 11 mV, R_{eff} nearly doubled, and the time constant (τ_m) increased by 60%. There was no significant difference in r_m or R_m (95% level, paired *t*-test).

The increase in intracellular K (K_i) consequent to shrinkage can cause the hyperpolarization observed. This increase in K_i appears to be inconsistent with the increase in R_i; Freygang et al. (1967) have suggested that the larger R_i may result from an increase in myoplasmic viscosity. The rise in C_m suggests that morphological changes might be present; however, data from fibers in which the tonicity was gradually raised suggest that these changes may be secondary. Increases in tonicity by 0.05 T up to 1.8 T caused a small increase in τ_m from 5.9 ± 2.6 (mean ± SD, n = 4) to 6.7 ± 2.7 ms. R_{eff} increased in all cases from 11.9 ± 4.2 to 21.0 ± 9.6 kΩ. In two of these four fibers we determined the membrane

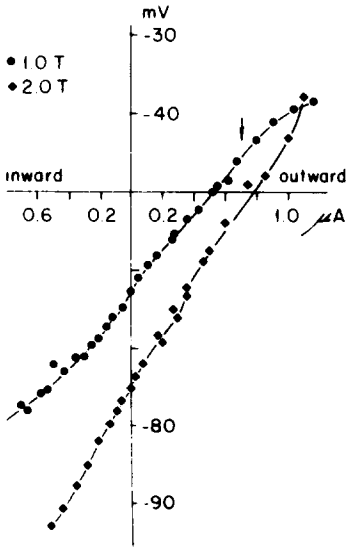

FIGURE 5. Effects of hypertonic saline on the current-voltage relationship of a single fiber. Data are plotted as the voltage deflection at the end of current pulses of 40-ms duration. Visually observed onset of tension in isotonic saline is marked by the arrow. In the hypertonic solution, no tension was elicited in the range of membrane depolarization shown in the plot.

cable properties: R_m increased in both cases from average values of 288 to 437 Ω·cm²; R_i also increased from 121 to 189 Ω·cm; C_m, however, decreased from 28.9 to 23.0 μF/cm². The notable differences lie in the lack of effect on τ_m and C_m as compared to fibers subjected to abrupt changes in tonicity; further study would be necessary to establish a consistent change in R_m. τ_m also decreased with the stepwise changes in tonicity.

The changes in τ_m and C_m found with rapid increases in tonicity might suggest the occurrence of morphological changes; however, attempts to study this problem by electron microscopy were unsuccessful due to inadequate fixation of fibers in hypertonic solutions. Even so, such changes as might occur must be secondary since the inhibition of membrane Ca activation and contraction occurs regardless of whether the increase in tonicity is made rapidly or gradually. In addition, studies by Zollman et al. (1974) on crayfish muscle fibers have shown that there is no effect on electrical parameters in fibers with swollen T systems.

Hypertonic Solutions Containing Glycerol and Acetamide

There is evidence that glycerol and acetamide permeate the sarcolemma of striated frog muscle fibers, acetamide being more readily permeant (Caputo, 1968). Measurements of fiber diameter during exposure to hypertonic solutions containing 1 M of either nonelectrolyte extend these findings to the muscle fibers of *C. danae*. The fibers initially shrank and then returned to the control values while bathed in the hyperosmotic media. With acetamide the transient changes in volume were completed within 5 min; the induced alterations of membrane response to depolarizing pulses followed a similar time course.

With glycerol the volume changes occurred at a slower rate and therefore allowed more extensive investigation of the effects on membrane electrogenesis and tension development. The transience of the inhibitory effects of hyperosmotic glycerol solutions on membrane Ca activation could be observed more clearly in procaine-treated fibers. The records shown in Fig. 6 were taken from a fiber exposed throughout the experiment to 2 mg/ml procaine, a concentration which markedly reduces the mechanical output of the induced spikes. A propagated action potential recorded in 1.0 T is shown in Fig. 6 A. Exposure to a hyperosmotic glycerol solution blocked the spike electrogenesis for the initial 3 min (Fig. 6 B). A graded membrane response followed by delayed repolarization occurred at 7 min and the propagated action potentials reappeared at 20 min (Fig. 6 C, D). At this time the fiber volume had practically returned to its control value, as determined by measurements of fiber diameter under a high-power compound microscope. A hypertonic solution of the same tonicity (2.2 T), but with NaCl replacing glycerol, was then introduced in the experimental chamber. Inhibition of the spikes was effected within 2 min, while fiber volume changes were still minimal, and persisted during the 10-min exposure to this solution; the spikes reappeared upon replacement of the fiber in isotonic saline (Fig. 6 F, G). A second exposure to the hyperosmotic glycerol solution (Fig. 6 H) caused a pattern of responses similar to that observed during the first treatment with this solution.

When fibers that had been equilibrated in hyperosmotic glycerol solutions were transferred to sucrose-containing solutions of the same tonicity, blockade of the procaine-induced spikes appeared only after 10 min. Measurements of fiber diameter indicated that fiber shrinkage due to the redistribution of glycerol was occurring at the onset of the blockade of the spikes.

Returning the fibers directly to isotonic saline after the Ca-dependent electrogenesis had recovered during exposure to hyperosmotic glycerol solutions resulted in fiber injury within 10 min.

DISCUSSION

The present experiments indicate that hypertonic solutions inhibit both the normal graded electrogenesis and the Ca-induced spikes in muscle fibers of *C. danae*. Depolarizing electrogenesis in crab fibers reflects the interplay of the voltage-dependent increases in Ca and K conductances (Mounier and Vassort, 1975). The inhibitory effect of hypertonicity could therefore result either from depression of membrane Ca activation or from enhancement of K activation. A role for Cl in the blockade of the depolarizing electrogenesis is unlikely in view of the observed inhibition of procaine spikes in hypertonic Cl-free saline.

The possibility that the blockade of the graded responses and spikes is due to increased activation of K conductance is unlikely for two reasons: first, the current-voltage relationship indicated that hypertonic solutions increase R_{eff} and do not enhance delayed rectification (Fig. 5); second, hypertonic solutions

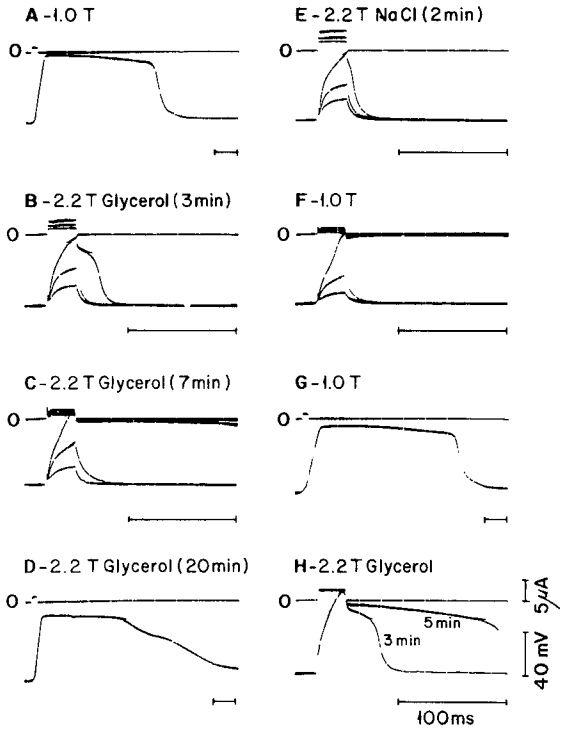

FIGURE 6. Effect of hyperosmotic glycerol saline on procaine (2 mg/ml) spikes in an isolated muscle fiber. In each panel, top records show currents and lower traces show the membrane potential. In panels A, D, and G the current electrode was inserted into the middle of the fiber and the recording micropipette was impaled near the tendon end of the fiber. In the other panels the micropipettes were impaled within 50 μm of one another in the middle of the fiber. A, procaine-induced spike in isotonic saline; B, C, and D, records taken during exposure to hyperosmotic glycerol solution; E. blockade of the spikes within 2 min of exposure to a hypertonic saline prepared by addition of NaCl; F, G, recovery of spike electrogenesis upon returning the fiber to the isotonic saline for 3 (F) and 5 min (G); H, superposed records obtained after 3 and 5 min, respectively, of a second exposure to the hyperosmotic glycerol solution. The horizontal calibration bars in all records indicate 100 ms.

inhibited the regenerative membrane responses obtained by treatment with agents which block K-conductance such as TEA, procaine, and Ba ions.

Inhibition of Membrane Ca Activation

If one excludes the possibility of increased activation of K or Cl conductances in hypertonic media, the most likely mechanism underlying the inhibition of the depolarizing electrogenesis is direct depression of the inward Ca current. The

transmembrane Ca current (I_{Ca}) at a given membrane potential (E_m) can be expressed by:

$$I_{Ca} = G_{Ca} (E_m - E_{Ca})$$

where G_{Ca} is the potential-dependent membrane Ca conductance and E_{Ca} is the equilibrium potential for Ca ions. There are two possibilities which might account for decreased I_{Ca}: a decrease in the driving force ($E_m - E_{Ca}$) or a decrease in G_{Ca}. The first of these appears less likely, but cannot be excluded as a possibility, since (*a*) the fibers generally hyperpolarized during treatment with hypertonic solutions, and (*b*) decrease in E_{Ca} due to increase in the sarcoplasmic free Ca concentration caused by fiber shrinkage or by release from intracellular stores (Lannergren and Noth, 1973) appears to have been small, since no tension was observed at tonicities where the membrane responses were inhibited. Also, a fivefold increase in Ca_0 did not reverse the effects of hypertonic solutions; however, here the effects of increased Ca_0 on E_{Ca} might have been counteracted by the resultant elevation of the external ionic strength (see below).

Since the inhibition of the Ca-dependent electrogenesis persisted during prolonged exposures to hypertonic media prepared by addition of nonpermeant solutes, but was transient when glycerol or acetamide was used, the effects on G_{Ca} could be due in part to a change accompanying fiber shrinkage, such as an increase in intracellular ionic strength. The effect of the latter might be explained tentatively by changes in the electric field across the membrane. It is assumed that the membrane surface contains fixed negative charges whose density will create a potential difference between the solution immediately at the membrane and at some more distant point; these surface potentials will not contribute to the membrane potential measured with microelectrodes but will affect the "effective" potential drop across the membrane and, consequently, the voltage-dependent ionic conductance channels (Baker et al., 1964; Chandler et al., 1965; Lorkovič, 1967). Increase in the internal ionic strength could affect the threshold for activation of ionic conductances—G_{Ca} in these fibers—through reduction of the electric field generated by the fixed charges, causing an "effective" membrane depolarization. In the case of Na currents in squid axon (Chandler et al., 1965), the threshold is shifted toward more negative values of the measured membrane potential; however, our experiments showed instead an increase in the threshold for Ca activation. We suggest that the "effective" depolarization postulated to occur in shrunken fibers may cause inactivation of G_{Ca}. Mounier and Vassort (1974) have proposed a similar mechanism for the inhibition of Ca spikes in crab fibers bathed in Cl-free media. Although conditioning hyperpolarization reactivated G_{Ca} in these investigators' experiment with *Carcinus maenas*, it failed to reverse the blockade of Ca spikes in hypertonic solutions. This might be due to differences in the voltage dependence of the availability of the Ca channel in the two experimental conditions. If the conductance of the Ca channels is appreciable in the resting membrane, its inactivation by elevated internal ionic strength might also contribute to the hyperpolarization observed in hypertonic media.

In fibers equilibrated with hyperosmotic glycerol, transfer to hypertonic NaCl solutions resulted in blockade of the procaine-induced spikes before changes in

fiber volume could be observed. If instead the fibers were transferred to a hyperosmotic sucrose medium, the blockade was observed only after fiber shrinkage due to the redistribution of glycerol. This difference might be due to changes in external ionic strength. On the assumption that fixed negative surface charges contribute to the transmembrane potential, the elevated external ionic strength in hypertonic NaCl saline could cause an "effective" hyperpolarization. This might result in an increase in the measured threshold for activation of G_{Ca}. A similar interpretation has been proposed for the increase in threshold for K-induced contractures in frog muscle (Lorkovič, 1967). An effective hyperpolarization could also block the procaine-induced spikes through inactivation of G_{Ca} since, in crab fibers, conditioning hyperpolarizing pulses decrease the availability of the Ca channels (Mounier and Vassort, 1975). An alternative interpretation is that the blockade of the Ca spikes in the glycerol experiments results from morphological changes in the tubular system. Although transfer to hypertonic NaCl solution caused no obvious change in the appearance of the *C. danae* fibers, those of *C. maenas* submitted to the same experimental procedure became cloudy and opaque immediately upon exposure to the hypertonic NaCl solution: within 3 min the sarcomeres were no longer visible (Suarez-Kurtz, unpublished observations). This was not observed when the fibers were transferred from glycerol- to sucrose-containing solutions. The changes observed in hypertonic NaCl saline are similar to those described by Reuben et al. (1967) for crayfish fibers with swollen T tubules. If similar although less extensive morphological changes of the internal membrane system occur in the fibers of *C. danae,* this may affect membrane excitability and cause blockade of the procaine-induced spikes in the absence of changes in fiber volume and intracellular ionic strength. Further experiments are necessary to examine this possibility.

Morphological changes may also occur in experiments with nonpenetrating solutes and contribute to the blockade of the Ca-dependent membrane responses; indeed, the increase in C_m is consistent with changes in the tubular system. However, it appears that this is not the main factor involved since the blockade was also observed when the tonicity was gradually increased, and any morphological changes in the tubular system as reflected by effects on C_m were minimized

Hypertonic Solutions and Excitation-Contraction Coupling

Activation of membrane Ca conductance is required for excitation-contraction coupling (ECC) in crustacean muscle fibers (Reuben et al., 1967; Suarez-Kurtz et al., 1972; Suarez-Kurtz and Reuben, 1975), and therefore its inhibition by hypertonic solutions can account for the depression of contractile responses to membrane depolarization induced either by outward current pulses or by elevated K_0. Since vigorous contractures by caffeine could still be elicited at tonicities which nearly abolished the contractions associated with membrane depolarization, it appears that the direct effect of increased intracellular ionic strength on the contractile proteins (Howarth, 1958; Podolsky and Sugi, 1967; April et al., 1968) is not the major factor involved in the uncoupling action of hypertonic solutions in the present experiments. Direct effects of ionic strength and viscosity on the contractile apparatus must be present, however, and

probably are more effective at tonicities greater than those which we have used.

The dissociation of depolarizing electrogenesis and tension development observed in the presence of Ba (Fig. 5) might indicate a different sensitivity, to the inhibitory effects of hypertonicity, of Ca inward currents as compared to other divalent cations. Greater sensitivity of Ca currents, as compared to other divalent cations, to blockade by hypertonicity has also been reported for cardiac muscle (Hermsmeyer et al., 1972).

We wish to thank Dr. John P. Reuben for a valuable critical review of the original manuscript. This work was supported in parts with grants from CNPq (TC-16.272 and TC-16.897) and from Conselho de Ensino para Graduados, Universidade Federal do Rio de Janeiro. Dr. Sorenson was a Visiting Scientist sponsored by CNPq (TC-16.841).

Received for publication 2 March 1976.

REFERENCES

APRIL, E., P. W. BRANDT, J. P. REUBEN, and H. GRUNDFEST. 1968. Muscle contraction: the effect of ionic strength. *Nature (Lond.)*. **220:**182.

ASHLEY, C. C., and E. B. RIDGWAY. 1970. On the relationships between membrane potential, calcium transient and tension in single barnacle muscle fibres. *J. Physiol. (Lond.)*. **209:**105.

BAKER, P. F., A. L. HODGKIN, and H. MEVES. 1964. The effect of diluting the internal solution on the electrical properties of a perfused giant axon. *J. Physiol. (Lond.)*. **170:**541.

CAPUTO, C. 1966. Caffeine- and potassium-induced contractures of frog striated muscle fibers in hypertonic solutions. *J. Gen. Physiol.* **50:**129.

CAPUTO, C. 1968. Volume and twitch tension changes in single muscle fibers in hypertonic solutions. *J. Gen. Physiol.* **52:**793.

CHANDLER, W. K., A. L. HODGKIN, and H. MEVES. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol. (Lond.)*. **180:**821.

FATT, P., and B. KATZ. 1953. The electrical properties of crustacean muscle fibres. *J. Physiol. (Lond.)* **120:**171.

FREYGANG, W. H., JR., S. I. RAPOPORT, and L. D. PEACHEY. 1967. Some relations between changes in the linear properties of striated muscle fibers and changes in ultrastructure. *J. Gen. Physiol.* **50:**2437.

GIRARDIER, L., J. P. REUBEN, P. W. BRANDT, and H. GRUNDFEST. 1963. Evidence for anion permselective membrane in crayfish muscle fibers and its possible role in excitation-contraction coupling. *J. Gen. Physiol.* **47:**189.

GORDON, A. M., and R. E. GODT. 1970. Some effects of hypertonic solutions on contraction and excitation-contraction coupling in frog skeletal muscle. *J. Gen. Physiol.* **55:**254.

HERMSMEYER, K., R. RULON, and N. SPERELAKIS. 1972. Loss of the plateau of the cardiac action potential in hypertonic solutions. *J. Gen. Physiol.* **59:**779.

HODGKIN, A. L., and P. HOROWICZ. 1957. The differential action of hypertonic solutions on the twitch and action potential of a muscle fibre. *J. Physiol. (Lond.)*. **136:**17P.

HODGKIN, A. L., and W. A. H. RUSHTON. 1946. The electrical constants of crustacean nerve fibres. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **133:**444.

HOMSHER, E., F. N. BRIGGS, and R. M. WISE. 1974. Effects of hypertonicity on resting and contracting frog skeletal muscles. *Am. J. Physiol.* **226:**855.

HOWARTH, J. V. 1958. The behavior of frog muscle in hypertonic solutions. *J. Physiol. (Lond.)*. **144:**167.

LANNERGREN, J., and J. NOTH. 1973. Tension in isolated frog muscle fibers induced by hypertonic solutions. *J. Gen. Physiol.* **61:**158.

LORKOVIĊ, H. 1967. The influence of ionic strength on potassium contractures and calcium movements in frog muscle. *J. Gen. Physiol.* **50:**883.

MOUNIER, Y., and G. VASSORT. 1974. Influence of Cl ions on the Ca-current in crab muscle fibre. *J. Physiol. (Lond.)*. **242:**92P.

MOUNIER, Y., and G. VASSORT. 1975. Initial and delayed membrane currents in crab muscle fibre under voltage-clamp conditions. *J. Physiol. (Lond.)*. **251:**589.

ORENTLICHER, M., and R. S. ORNSTEIN. 1971. Influence of external cations on caffeine-induced tension. Calcium extrusion in crayfish muscle. *J. Membr. Biol.* **5:**319.

PODOLSKY, R. J., and H. SUGI. 1967. The influence of external hypertonic solutions on the contractile mechanism of skeletal muscle fibers. *J. Gen. Physiol.* **50:**2496.

REUBEN, J. P., P. W. BRANDT, H. GARCIA, and H. GRUNDFEST. 1967. Excitation-contraction coupling in crayfish. *Am. Zool.* **7:**623.

SUAREZ-KURTZ, G. 1974. Inhibition of membrane calcium activation by neomycin and streptomycin in crab muscle fibers. *Pfluegers Arch. Eur. J. Physiol.* **349:**337.

SUAREZ-KURTZ, G., and J. P. REUBEN. 1975. Is a Ca current necessary for excitation-contraction coupling in skeletal muscle? *In* Concepts of Membranes in Regulation and Excitation. M. Rocha e Silva and G. Suarez-Kurtz, editors. Raven Press, New York. 41.

SUAREZ-KURTZ, G., J. P. REUBEN, P. W. BRANDT, and J. GRUNDFEST. 1972. Membrane calcium activation in excitation-contraction coupling. *J. Gen. Physiol.* **59:**676.

SUAREZ-KURTZ, G., and A. L. SORENSON. 1974. Effects of hypertonic solutions on membrane Ca activation in crab muscle fibers. *Ann. Acad. Brasil. Cienc.* **46:**669.

ZOLLMAN, J., A. EASTWOOD, J. P. REUBEN, and P. W. BRANDT. 1974. Ionic permeabilities and equilibria in the TTS of crayfish muscle. *Fed. Proc.* **33:**1402.