Caffeine- and Potassium-Induced Contractures of Frog Striated Muscle Fibers in Hypertonic Solutions

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ABSTRACT The effect of hypertonic solutions on the caffeine- and KCl-induced contractures of isolated fibers of frog skeletal muscle was tested. Hypertonic solutions, twice the normal osmotic strength, prepared by adding NaCl or sucrose, potentiate the caffeine-induced contractures. The fibers may develop tensions of 3.6 kg/cm^2 of fiber transverse section. The same hypertonic medium reduced the peak tension of KCl-induced contractures. Thus the hypertonic condition does not affect the contractile mechanism itself. These findings give further support to the view that the differential effect of hypertonic solution is on the excitation-contraction coupling mechanism. Extracellular calcium is not essentially required for the first few of a series of caffeine-induced contractures either in hypertonic or in isotonic solutions.

The depolarization of the muscle cell membrane is essential for the activation of the contractile mechanism under normal conditions (1–4). The mechanical response of muscle fibers may be reduced or abolished without interfering with the conduction of action potentials (5–8). However, caffeine activates the contractile mechanism of muscle fibers without inducing any relevant change in their resting membrane potentials (9). Furthermore, caffeine can induce contractures even in a completely depolarized muscle (9). Thus, caffeine seems capable of activating the contractile material of muscle fibers, through a mechanism that bypasses the steps of the excitation-contraction coupling (ECC) which depend on the depolarization of the fiber membrane.

Hodgkin and Horowicz (5) have shown that hypertonic solutions (2.5 times normal tonicity) abolish the twitch of a frog muscle fiber while its membrane is electrically excitable and conducts action potentials normally. It has been suggested that hypertonic solutions could act by impairing either the contractile material itself (10, 11) or one or more links of the ECC (10,

12, 13). Previous studies indicating that caffeine contractures are not affected by hypertonic solutions (14, 15) could contribute to the understanding of the differential action of hypertonic solutions on the electrical and mechanical responses of muscle fibers. The present experiments were carried out to extend further this information. The effects of hypertonic solutions on the caffeine-induced contractures and on the potassium-induced contractures were studied. Single fibers or small bundles of fibers were used in order to avoid diffusion delays.

MATERIAL AND METHODS

The leopard frog, *Rana pipiens*, has been used throughout this study. The animals were flown from Oshkosh, Wisconsin, kept in a large tank with running water, and used within a month of their arrival.

Single fibers, or small bundles of from two to six fibers arranged in a single layer, were dissected from the semitendinosus muscle, with the muscle immersed in a bath of curarized Ringer's solution (10^{-5} g of tubocurarin per ml). After dissection, the fibers were allowed to rest for a period of 30 to 60 min to detect any possible damage to the fibers. After this period, the fibers which appeared to be in good condition were tested for excitability with electrical stimuli delivered by a Tektronix 161 pulse generator. The fibers that gave visible response were mounted in a Lucite chamber similar to the one described by Hodgkin and Horowicz (16). The fibers were placed in a groove running longitudinally in the bottom of the chamber; one tendon was gripped by a clamp fixed in the groove, and the other tendon tied to the prolonged anode of an RCA 5734 transducer. The transducer was mounted on a micromanipulator and could be moved in order to stretch the fibers slightly beyond slack length. The groove in which the fibers lay was covered by a glass coverslide for most of the fiber length. A revolving tap allowed quick change of the experimental solutions. The solutions were withdrawn from the chamber by suction. Once mounted in the chamber, the fibers were allowed to rest for another period of approximately 30 min and then tested again for excitability. Stimulation of the fibers was carried out through two platinum wires cemented in the bottom of the groove. The transducer was connected to a conventional circuit and its output fed to an oscilloscope (Tektronix, 565; plug in unit 2A63), whose screen was photographed. The transducer was calibrated by means of known weights after each experiment.

Table I shows the composition of the various solutions used. The osmolality of the solutions was measured with a freezing point osmometer (Fiske osmometer). The normal Ringer solution, solution *a* in Table I, with an osmolality of 230 mOsM/kg of water, is considered isotonic. The osmolality of the other solutions is given relative to this one. The temperature varied between 19 and 21°C.

RESULTS

Caffeine Contractures

It has been found that different muscle fibers present various degrees of sensitivity to caffeine. There are two critical caffeine concentrations, which vary

from fiber to fiber: one is the threshold caffeine concentration necessary to induce a contracture and the other is the maximal concentration that does not produce obvious damage to the fiber. For the contracture elicited in isotonic medium the minimal and maximal caffeine concentrations used were 0.5 to 1.0 g/liter. For some fibers the 0.5 g/liter concentration was found to be subthreshold. However, in these cases the drug at this concentration acted to facilitate the production of a contracture elicited in a second trial with the same drug concentration. For the contractures produced in hypertonic medium, the minimal and maximal drug concentrations used were 0.1 and 0.75 g/liter. In this range of concentration the mechanical response of the fibers was not adversely affected by caffeine, although some structural changes have been observed in the fibers, even at relatively low caffeine concentrations (0.1 g/liter) (17).

TABLE I
COMPOSITION OF SOLUTIONS

Solution	NaCl	KCl	$CaCl_2$	Na_2HPO_4	NaH_2PO_4	Relative osmolality
			mM/liter			
(*a*) Normal (Ringer's)	115	2.5	1.8	2.15	0.85	1
(*b*) Hypertonic	230	2.5	1.8	2.15	0.85	1.9
(*c*) 40 K isotonic	77.5	40	1.8	2.15	0.85	1
(*d*) 40 K hypertonic	192.5	40	1.8	2.15	0.85	1.9
(*e*) 80 K isotonic	37.5	80	1.8	2.15	0.85	1
(*f*) 80 K hypertonic	152.5	80	1.8	2.15	0.85	1.9
(*g*) 0 Ca isotonic	115	2.5	—	2.15	0.85	~1
(*h*) 0 Ca hypertonic	230	2.5	—	2.15	0.85	~1.9

The caffeine contractures are expressed in terms of maximal tension instead of areas under the contractures, since some of the fibers relaxed spontaneously in the presence of the drug, while others needed withdrawal of the drug to relax. For the same reason the rate of relaxation of the fibers is not reported. As shown in Figs. 1 and 2, caffeine-induced contractures were not inhibited, but potentiated by hypertonic solution. During a contracture induced by 0.5 g/liter of caffeine, a single fiber developed a tension of 52 mg at a rate of 16 mg/sec, in isotonic medium (Fig. 1 A), while in hypertonic medium 88 mg of tension developed at a rate of 48 mg/sec (Fig. 1 B). After a fiber developed a potentiated contracture in response to caffeine in hypertonic medium it lost temporarily its capacity to produce a contracture in isotonic, but not in hypertonic, medium, and it needed a recovery period in a medium without caffeine to give a response (Fig. 1 C and Fig. 2 A).

Caffeine contractures in hypertonic solution occurred immediately after exposure to the hypertonic medium. It was found that hypertonic solution

markedly diminished the twitch tension in response to electrical stimuli (Fig. 2 B), as in the study of Hodgkin and Horowicz (5). Fig. 2 C shows that the response to caffeine is still present even though the fiber is maintained in the hypertonic medium for 10 min before eliciting it.

Similar results were obtained using sucrose instead of NaCl as an osmotic agent but not with some nonelectrolyte molecules of smaller size than sucrose (unpublished results).

Fig. 3 shows the potentiating effect of hypertonic solutions on the caffeine-induced contractures at different drug concentrations. These experiments

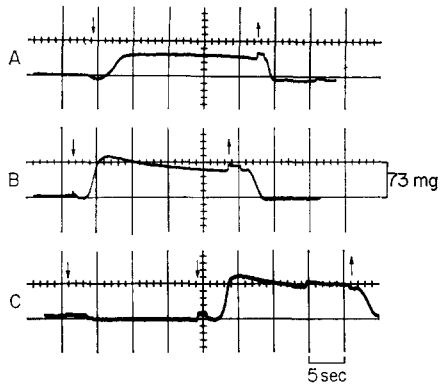

FIGURE 1. Oscilloscope records showing the effect of hypertonic solution on caffeine-induced (0.5 g/liter) contractures of a single muscle fiber. In this, and in the following figures, the solutions referred to are given in Table I. The arrows indicate the change of solutions as follows: Record A, fiber in solution *a*; change to solution *a* plus caffeine, then back to solution *a*. Record B, fiber in solution *a*; change to solution *b* plus caffeine, then back to solution *a*. Record C, fiber in solution *a*; change to solution *a* plus caffeine; change to solution *b* plus caffeine, then returned to solution *a*. Record B was obtained 10 min after record A. Record C was obtained immediately after record B. The artefacts in the records were caused by the sudden flow of solutions in the chamber, or by stopping the flow after solution change.

show that the contractile mechanism of the fibers is capable of generating maximal tensions in hypertonic medium. Assuming a mean diameter of 70 microns for these fibers, and taking the mean value of 135 mg of maximal tension per fiber as obtained from Fig. 3, a value of 3.6 kg of tension/cm^2 of fiber transverse section is obtained. This value is similar to the value found by Hodgkin and Horowicz for the maximal tetanic tension of this type of fibers from *Rana temporaria* (4).

The rate of development of tension during caffeine contractures, elicited in isotonic medium, has been found to increase with the drug concentration and consequently with the contracture tension. The mean values (± the standard error of the mean) of the slopes of the rising phase of the contractures induced

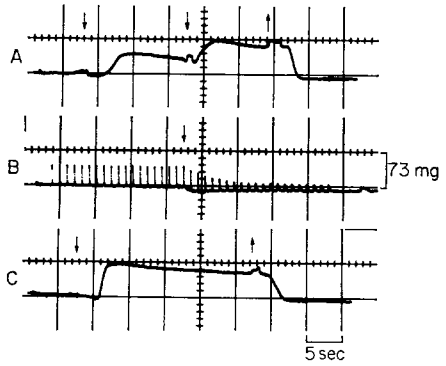

FIGURE 2. Oscilloscope records showing the effect of hypertonic solution on caffeine-induced (0.5 g/liter) contractures, and twitch tension of a single muscle fiber. Record A, fiber in solution *a*; change to solution *a* plus caffeine; change to solution *b* plus caffeine, then returned to solution *a*. Record B, fiber stimulated at 1 stimulus per sec in solution *a*, then changed to solution *b*. Record C, fiber in solution *b*; change to solution *b* plus caffeine, then to solution *a*. Same fiber as in Fig. 1. Record A of this figure was obtained 15 min after record C of Fig. 1. Record B was obtained 10 min after record A. Record C was obtained 10 min after record B.

by 0.5 g/liter, 0.75 g/liter, and 1.0 g/liter caffeine in isotonic medium, are respectively 20 ± 3 mg/sec (five fibers), 32 ± 6 mg/sec (six fibers), and 41 ± 5 mg/sec (six fibers). The mean value (± the standard error of the mean) of the slope of the rising phase of the contractures elicited by 0.5 g/liter of

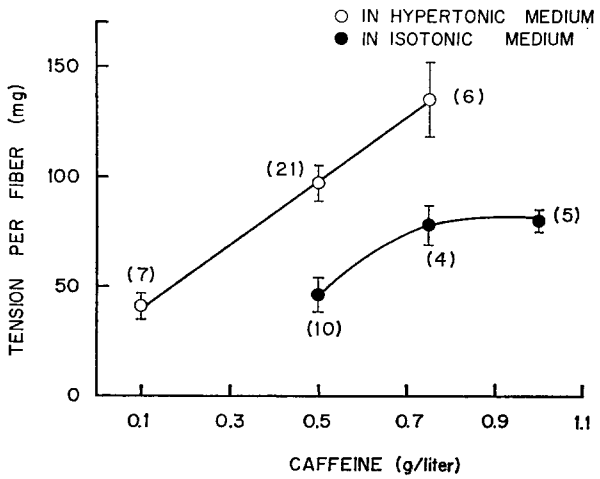

FIGURE 3. The relation between maximal contracture tension per fiber and caffeine concentration in isotonic and hypertonic media (solutions *a* and *b*, Table I). Each point represents the mean (±1 standard error of the mean) of tensions obtained with different fibers. When bundles were used the number of fibers in the bundle served to normalize the tension per fiber. The number of experiments performed is given in parentheses.

caffeine in hypertonic medium was 51 ± 7 mg/sec (six fibers). It seems fair to compare this last value with the mean value of the rate of development of tension during the contractures induced by 1.0 g/liter of caffeine in isotonic medium, since the maximum tensions developed during the contractures elicited in the two conditions are also comparable. The values of 51 ± 7 mg/sec and 41 ± 5 mg/sec are not statistically different ($P = 0.30$).

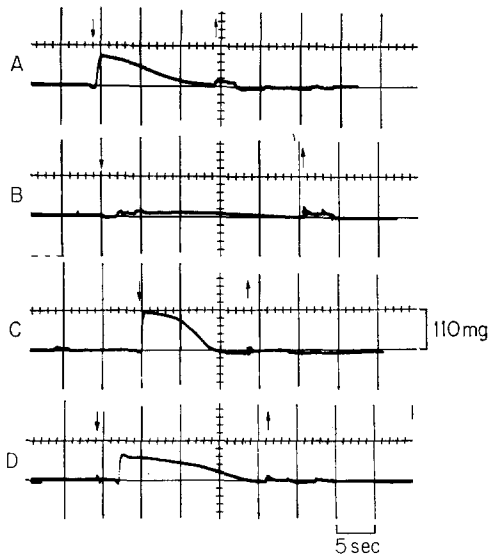

FIGURE 4. Oscilloscope records showing the effect of hypertonic solutions on the potassium contractures of a single fiber. An interval of 10 min separates the following records: Record A, fiber in solution *a*, change to solution *c*, then back to solution *a*. Record B, fiber in solution *a*; change to solution *d*, then back to solution *a*. Record C, fiber in solution *a*, change to solution *e*, then to solution *a*. Record D, fiber in solution *a*, change to solution *f*, then returned to solution *a*.

Potassium Contractures

The effect of hypertonic solutions on the potassium-induced contractures is shown in Fig. 4. In response to a potassium concentration of 40 mM/liter, a single fiber developed a tension of 76 mg in isotonic medium (Fig. 4 A) and of 19 mg in the hypertonic medium (Fig. 4 B). When the potassium concentration was 80 mM/liter the contracture tensions obtained in isotonic and hypertonic media were 116 mg (Fig. 4 C) and 77 mg (Fig. 4 D) respectively. The same experiments were repeated with four different fibers with similar results. These results are in agreement with those of Hodgkin and Horowicz, who found that a muscle fiber might still develop some tension in response to a concentration of potassium of 270 mM/liter after a solution of the same tonicity had abolished the twitch (5).

Effect of External Calcium Deprivation

It has been shown that caffeine contractures occur in muscles equilibrated in an isotonic medium free of calcium (18). The following experiments were performed to explore whether deprivation of calcium affected the caffeine contractures in hypertonic medium.

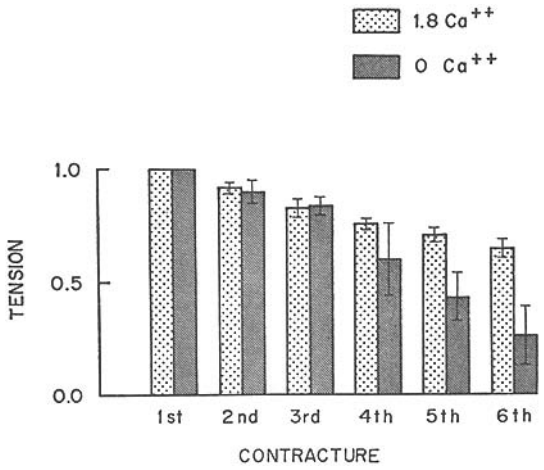

FIGURE 5. Responses of single fibers to six successive exposures in caffeine-containing solutions (0.5 g/liter). The dotted bars are the mean of five experiments performed in solution *b* of Table I. The cross-hatched bars are the mean of four experiments performed in solution *h*. Each fiber was incubated for 30 min in the respective solution before eliciting the first contracture, and was allowed to rest for 10 min between contractures. The vertical bars represent the mean values ± the standard error of the mean. The results are expressed relative to the mean values of the first contractures for each group. These were 128 ± 16 for the fibers incubated in solution *b* and 115 ± 11 for the fibers incubated in solution *h*.

Single fibers were incubated in isotonic media free of calcium during 30 min before applying caffeine at a concentration of 0.5 g/liter. The mean value (± standard error of the mean) of the maximum tension developed during contractures of five fibers kept in the calcium-free isotonic solution (solution *g* of Table I) was 73 ± 12 mg; while the mean value (± standard error of the mean) of the maximum tension developed during the contractures of four fibers kept in the calcium-free hypertonic solution (solution *h* of Table I) was 115 ± 11 mg. These values appear to be slightly greater than the corresponding values obtained with fibers incubated in media with normal calcium concentration. This suggests that addition of calcium to the bathing medium is not essential for the production of caffeine-induced contractures in isotonic or hypertonic media.

Since there is evidence that calcium plays an important role in the caf-

feine-induced contractures (18–20), it is possible that some of the intracellular calcium intervenes in the contractures observed in the calcium-free media. Recently, deposits of intracellular calcium have been localized in the interior of the muscle fibers at the level of the sarcoplasmic reticulum (21, 22).

The response of single fibers to repeated caffeine exposures in hypertonic media with and without calcium was also studied. Fig. 5 shows that the calcium-free condition does not impair the first three caffeine-induced contractures, but that the responses decrease afterwards more rapidly in these fibers than in those kept in the medium with normal calcium content.

Effect of Procaine

The effect of procaine on the caffeine contractures induced in hypertonic medium was tested since it is known (20, 23) that procaine prevents caffeine from inducing contractures in isotonic media. Four experiments showed that 10 mM/liter of procaine inhibit also the caffeine contractures elicited in hypertonic medium.

DISCUSSION

Hypertonic solutions abolish or attenuate the twitch response of muscle fibers without affecting their electrical activity (5, 6). Howarth has suggested that the contractile elements of the fibers immersed in hypertonic solution could not shorten adequately during the active state because of an increase in the viscosity of the muscle fiber interior (11). The results of the present work indicate that hypertonic solutions potentiate the caffeine-induced contractures. The fibers exposed to caffeine in hypertonic media develop a maximum tension of 3.6 kg/cm^2 of fiber transverse section. The rates of tension development are not significantly different for caffeine contractures elicited in isotonic or hypertonic media under conditions in which tensions of comparable magnitude are developed (see Results). This would not be expected if the raised viscosity of the fibers, caused by the hypertonic medium, had an adverse effect on the contractile mechanism.

The present study provides additional evidence for the view that the effect of hypertonic solutions is on the excitation-contraction coupling mechanism. Hypertonic solutions interfere with responses to electrical stimuli and to potassium depolarization. However, they do not interfere with caffeine responses which are not mediated by depolarization of the cell membrane (9). The inhibition by hypertonic solutions may be due to an effect on the mechanism by which the depolarization of the membrane is linked to the activation of the contractile material. (For references on such a mechanism see references 24 and 25.) This is compatible with the suggestion (13) that hypertonic solutions inhibit a linking chemical process at or near the plasma membrane.

When fibers are bathed in hypertonic media of the strength used in the present experiments, the fiber volume is reduced to 0.6 times its original value (26–28). We have found that the diameter is reduced to about 0.8 times its original value during the first minute of incubation in this solution (unpublished results). Thus, hypertonic solutions should produce a decrease in the time of diffusion necessary for caffeine to reach the center of the fiber. The cell membrane constitutes no barrier to caffeine movement into the cell (29). However, it has been shown (9) that the drug evokes a response only when applied to the outer surface of the membrane. Huxley (30) has shown that ferritin molecules, 100 A in diameter, can move in the transverse tubules of the sarcoplasmic reticulum. Thus, it is tempting to suggest that caffeine molecules, whose major diameter is about 10 A, can move also in the tubules and reach a region near the contractile material, where they could act.

Caffeine increases the outflux of calcium from whole sartorius muscle (31). More recently it has been shown that caffeine releases calcium from, and inhibits the uptake of calcium by, isolated elements of sarcoplasmic reticulum (32). Though changes occur in the elements of the reticulum (33, 34) when muscle fibers are soaked in a hypertonic medium, it may be assumed that these changes do not interfere with the diffusion of caffeine along the transverse tubules of the reticulum. There is the possibility that changes in the structure of the sarcoplasmic reticulum could facilitate the action of caffeine in inducing contractures. The increase in the rate of tension production observed in the caffeine-induced contractures in hypertonic medium is in agreement with the decrease in fiber diameter and the estimated reduction in diffusion time.

In hypertonic and in isotonic solutions, extracellular calcium seems unnecessary for the response of the fibers to caffeine application. However, when successive responses are elicited, the contracture tension decreases faster in media with no calcium.

External calcium could serve as a source to replenish the exhausted supply of intracellular calcium, which may be sufficient only for the first few contractures elicited in the calcium-free medium.

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