Calcium and Magnesium Contents and Volume of the Terminal Cisternae in Caffeine-treated Skeletal Muscle

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ABSTRACT   (a) The effects of caffeine on the composition and volume of the terminal cisternae (TC) of the sarcoplasmic reticulum (SR) in frog skeletal muscle were determined with rapid freezing, electron microscopy, and electron probe analysis. (b) Caffeine (5 mM) released ~65% of the Ca content of the TC in 1 min and 84% after 3 min. The release of Ca from the TC was associated with a highly significant increase in its Mg content. This increase in Mg was not reduced by valinomycin. There was also a small increase in the K content of the TC at 1 min, although not after 3 min of caffeine contracture. (c) On the basis of the increase in Mg content during caffeine contracture and during tetanus (Somlyo, A. V., H. Gonzalez-Serratos, H. Shuman, G. McClellan, and A. P. Somlyo, 1981, J. Cell Biol., 90:577–594), we suggest that both mechanisms of Ca release are associated with an increase in the Ca and Mg permeability of the SR membranes, the two ions possibly moving through a common channel. (d) There was a significant increase in the P content of the TC during caffeine contracture, while in tetanized muscle (see reference above) there was no increase in the P content of the TC. (e) Mitochondrial Ca content was significantly increased (at 1 and at 3 min) during caffeine contracture. Valinomycin (5 μM) blocked this mitochondrial Ca uptake. (f) The sustained Ca release caused by caffeine in situ contrasts with the transient Ca release observed in studies of fragmented SR preparations, and could be explained by mediation of the caffeine-induced Ca release by a second messenger produced more readily in intact muscle than in isolated SR. (g) The TC were not swollen in rapidly frozen, caffeine-treated muscles, in contrast to the swelling of the TC observed in conventionally fixed, caffeine-treated preparation, the latter finding being in agreement with previous studies. (h) The fractional volume of the TC in rapidly frozen control (resting) frog semitendinosus muscles (~2.1%) was less than the volume (~2.5%) after glutaraldehyde-osmium fixation.

It is well known that caffeine can cause contraction of skeletal muscle without depolarization (2, 11, 47) through the release of calcium from the sarcoplasmic reticulum (75, 76; for review, see reference 20), and swelling of the sarcoplasmic reticulum (SR)[1] has been observed in electron micrographs of muscles that were fixed conventionally (with glutaraldehyde-osmium) during caffeine contracture (31, 72, 80). Recently, it has become possible to quantitate in situ with electron probe analysis not only the amount of Ca released, but also

the associated movement of other ions (K, Mg) into the SR during tetanus (43, 65, 66). In addition, the preservation of tissues through rapid freezing, rather than by the use of liquid fixatives, has revealed that swelling of the SR in conventionally fixed preparations can be due to fixation artifacts, rather than reflecting its volume in situ (28, 66).

The purpose of the present study was to determine the amount of Ca released from the SR during caffeine contracture, the nature of associated ion movements, and whether the caffeine-induced swelling previously observed in the SR after liquid fixation can also be observed in rapidly frozen (28, 66, 73, 74) muscles. In the present study, we report changes in the composition of the terminal cisternae (TC) of

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[1] Abbreviations used in this paper: EDL, extensor digitorum longus IV (muscle); SEMI, semitendinosus (muscle); SR, sarcoplasmic reticulum; TC, terminal cisternae; T-tubule, transverse tubule.

the SR and in mitochondria detected with electron probe x-ray microanalysis of cryosections of caffeine-treated muscles and volume measurements of the TC in rapidly frozen, freeze-substituted muscles. Some of the preliminary results have been presented to the Biophysical Society (79, 81).

MATERIALS AND METHODS

Bundles of 20–30 fibers with both tendons attached were dissected from frog semitendinosus (SEMI) muscle of *Rana pipiens*. The extensor digitorum longus IV (EDL) muscle was also used as an intact bundle. The dissection was done at room temperature and the bundles were allowed to rest >2 h in order to check for the presence of damaged fibers. The bundles were then stimulated to produce a twitch; poorly responsive or opaque bundles were discarded.

The composition of Ringer's solution in millimolar was: NaCl, 115; $CaCl_2$, 1.8; KCl, 2.5; Na_2HPO_4, 2.1; NaH_2PO_4, 0.9; with a pH of 7.2 (1). Caffeine Ringer's solution was made by adding various concentrations (1.0–5.0 mM) of caffeine to normal Ringer's solution. BSA, 4 g/100 ml (fatty acid free, Sigma Chemical Co., St. Louis, MO), was added to each solution to minimize the formation of ice crystals in the extracellular space. The 4% BSA Ringer's solution was adjusted to pH 7.2 with 0.5 N NaOH solution.

The SEMI or EDL muscle was mounted on a low-mass, stainless steel mesh holder specially designed for processing rapidly frozen and freeze-substituted preparations. We put the bundle, at a slightly stretched length (sarcomeres ~2.6–2.8 μm), on the central region of the domed portion of the holder by hooking both tendons on steel wires (66) and connected one end to a force transducer for monitoring tension. We froze bundles of control (resting) muscles and others exposed to caffeine for 1 or 3 min, respectively, at room temperature (23–25°C) by removing the beaker containing the solution and shooting Freon 22, that had been supercooled to −165 ± 5°C with liquid nitrogen, up to the bundle at 80–100 cm/s (41, 43, 65, 66). Tension was monitored throughout (Fig. 1). The frozen muscle bundle with the holder was transferred into cold, dry acetone for freeze substitution (28, 73, 74). These samples were kept in a deep freezer (−80°C) for at least 3 d, then gradually warmed and fixed with 10% OsO_4 in dry acetone, followed by block staining with uranyl acetate in methanol, dehydration, and embedding in Spurr's resin (67). The well-frozen portion of the muscle is generally where the bundle first makes contact with the supercooled Freon. Preliminary (transverse) sections were obtained from the block to check the best frozen region, and the block was then rearranged for longitudinal sectioning. Ultrathin (40–60 nm) sections were obtained on an LKB (LKB Producter, Bromma, Sweden) ultramicrotome, stained with lead citrate, and observed in a Zeiss EM109 electron microscope (Zeiss Co., Ltd., Federal Republic of Germany). Preparations (with and without caffeine and 4% BSA) were also fixed conventionally with 1% glutaraldehyde and 1% OsO_4 in 0.1 M phosphate buffer solution (80). Fixation of the caffeine-treated preparations was started after 1-min exposure to the drug.

Electron micrographs were taken from the control and paired caffeine-treated muscles (2.0 and 5.0 mM) at original magnifications of 7,000 and 12,000. These magnifications were checked with a calibration grid (No. 6002; 54,864 lines per inch; Ernest F. Fullam Inc., Schenectady, NY) before viewing
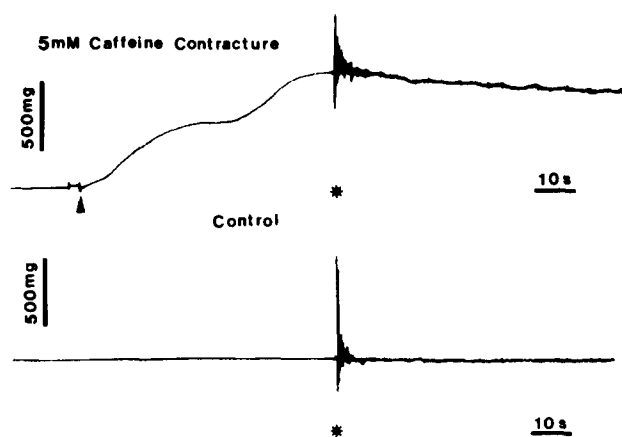

FIGURE 1 Tension traces recorded before freezing: a caffeine contracture (*upper trace*) and a control (*lower trace*) frog semitendinosus muscle. Tension development induced by an application of 5 mM caffeine (arrowhead) continues with two peaks of the contracture curve in this case. After 1 min of the caffeine contracture, the supercooled Freon was shot up to the bundle (asterisk) (see text).

the specimens. Prints were made at three times the original magnification. Volume of the TC was measured using a digital planimeter (57) and also by point-counting analysis (14, 17, 50, 77) from at least 12 electron micrographs for each frog muscle. This was run as a blind study. Three to four blocks were obtained from each animal, one or two grids taken from each block and two or three micrographs from each grid. The length of the A bands was measured in resting muscles showing the least evidence of ice crystals, because of the marked distortion of the sarcomere pattern during caffeine contracture (see Results).

Selection of fields to be photographed in freeze-substituted preparations was based solely on finding the regions showing minimal (<10 nm) ice crystal formation at the A-I junction. Any systematic sampling error introduced by the inevitable selection of the most superficial (i.e., best frozen) areas of the fibers (28, 65, 73) was the same for both the control and caffeine-treated preparations.

Cryosections, 100–200 nm thick, were obtained with an LKB cryoultramicrotome using toluene as a low temperature cement for fixing frozen preparations to the chuck of the cryotome (38) at a specimen temperature of −115°C and a glass knife temperature of −105°C; cryosections were freeze-dried for electron probe analysis as previously described in detail by Somlyo et al. (65, 66). After cryosectioning, the remainder of the frozen bundle was prepared for freeze substitution. Electron probe analysis was done on a Philips EM400 transmission electron microscope (Philips Corp., The Netherlands) fitted with a goniometer stage, a 30-mm² Kevex Si(Li) x-ray detector (Kevex Corp., Foster City, CA) and a multichannel analyzer interfaced to a PDP11/34 computer system (Digital Equipment Corp., Marlboro, MS). The methods used for quantitation and statistical analysis have been described in detail (42, 60, 65, 66). The accuracy of K and Ca measurements was further improved by including the first and second derivatives of the K x-ray peaks in the multiple least squares fit (for details, see reference 42). The underestimate of concentrations owing to the presence of higher atomic number elements (i.e., other than C, N, O, H) approximates, within the statistical error of measurement, 7–11% (see also reference 61), and does not affect the calculated differences in elemental concentrations.

RESULTS

The main feature of caffeine-induced contractures is illustrated in the upper trace of Fig. 1. At concentrations of ≥2.5 mM, caffeine contracture was obtained, showing a threshold similar to that observed in other muscles (2, 11, 47, 72, 80). With 5 mM concentrations of caffeine, the tension shows several (usually two) peaks (Fig. 1) before ending in a maintained plateau (47, 72). The plateau was usually reached by the end of the first minute of the caffeine contracture and was ~50–70% of the maximal tetanic tension. However, these experiments were not conducted on single fibers but on bundles in which activation by caffeine is non-uniform due to diffusional delays. Therefore, a direct comparison of the caffeine contractures with the uniformly (electrically) activated tetanic forces studied in previous experiments (65) is not feasible. Rapid freezing of the bundle during caffeine contracture after a 1-min application of the drug is also shown in Fig. 1. The rapid transient represents the shooting up of the supercooled Freon 22 to the bundle (asterisk in Fig. 1), and this is followed by a small noise in the tension record due to a mechanical artifact caused by contact of the metal wire holding the muscle and transducer (65).

Structural Effects of Caffeine and the Volume of the TC

Electron micrographs of rapidly frozen and freeze-substituted muscles, which were taken from the EDL and SEMI muscles, are illustrated in Figs. 2 and 3. In the normal controls (Fig. 2), the regular sarcomere pattern and the organization of the organelles (sarcoplasmic reticulum, mitochondria, nuclei, lipid droplet, glycogen particle) was similar to that of the conventionally fixed preparations (see also references 28 and 65). The extent of the ice crystal–free region varied from
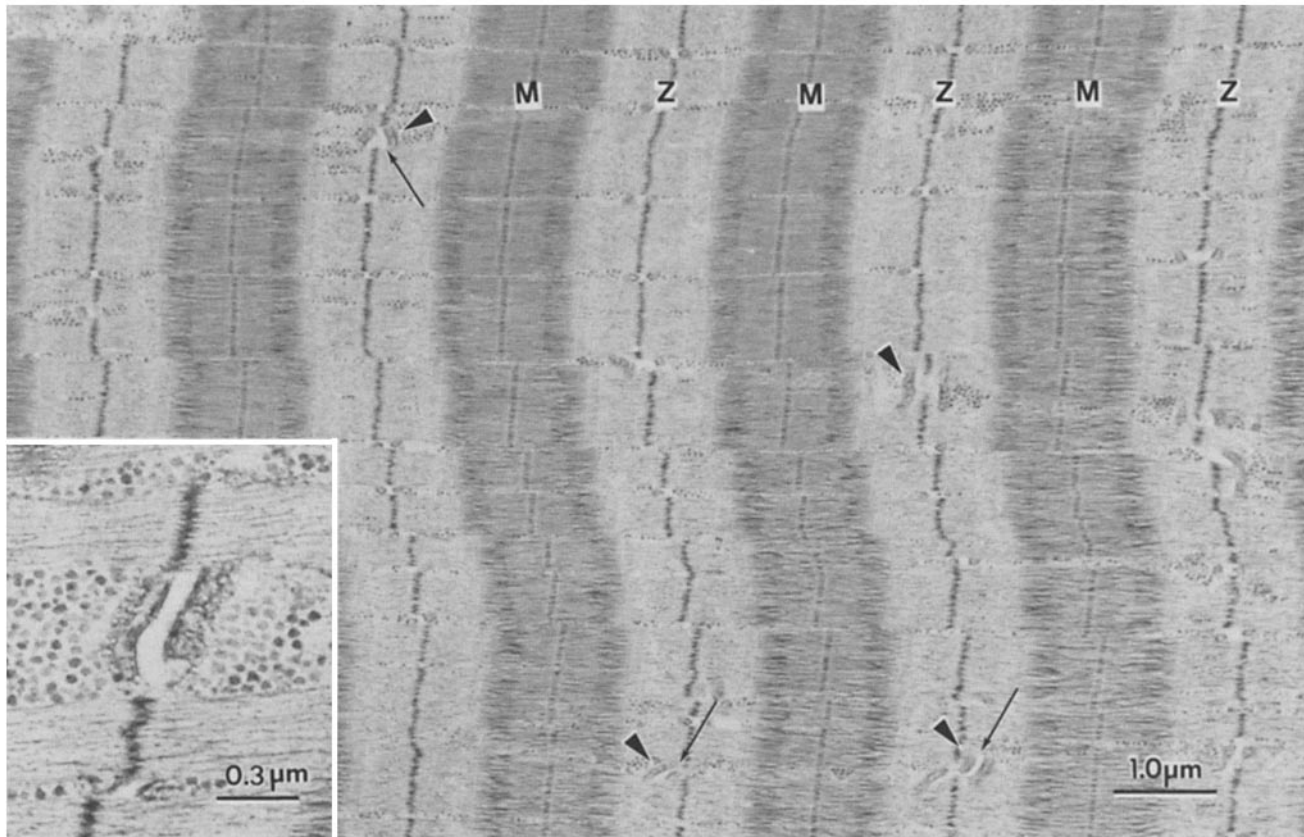
FIGURE 2 Longitudinal section of a rapidly frozen and freeze-substituted extensor digitorum longus muscle incubated with 4% BSA Ringer's solution. The fine structure is preserved in well-frozen portions (upper part of panel), and is similar to that seen after chemical fixation. TC (arrowheads) and T-tubules (arrows) with tiny periodic dots on the inside can be seen (*inset*). *M*, M line; *Z*, Z band. × 13,600; (*inset*) × 35,500.

preparation to preparation, but ice crystals were always absent when the fiber was cut within <7 μm of the surface. The depth of well-frozen tissue can vary from 1 to 20 μm, depending on such factors as the extent of blotting away excess Ringer's solution, the presence of connective tissue over the muscle, and the location of the surface of the fiber that makes first contact with the coolant. The lumen of the TC contained electron-opaque material, presumably calsequestrin (33, 48). A narrow space, presumably representing the junctional gap (26, 27, 64, 78), was seen at higher magnification (insets in Figs. 2 and 3), but frequently this region was collapsed. The "foot processes" (25), "bridging structures" (64), or "pillars" (16) were not seen, but occasionally, small periodic structures were found projecting into the transverse tubule (T-tubule). These may represent collapsed "bridging structures," possibly owing to the effect of the lipid solvent (acetone) used for freeze substitution. The length of the A band was 1.3 ± 0.12 μm (mean ± SD, *n* = 515, 55 micrographs from 15 frogs), and therefore, given an in vivo A band length of 1.5–1.6 μm, freeze substitution and associated preparatory methods (embedding, sectioning, etc.) resulted in an overall linear shrinkage of ~11–17%, compared with the 15–20% shrinkage reported by Brown and Hill (8).

Irregularities in sarcomere organization varying from regions of overstretched sarcomeres to supercontraction were also observed in freeze-substituted muscles that were rapidly frozen during caffeine contracture (Fig. 3; see also Fig. 5). Moreover, asymmetrical sarcomeres, in which thick filaments appeared to have been pulled towards one Z line, were present

in many myofibrils (15, 55). The TC retained a granular content and normal shape during caffeine contracture, and no detectable swelling was present in freeze-substituted preparations exposed to 5.0 mM caffeine, although the change in the TC volume (swelling) was marked in conventionally fixed preparations (Fig. 4).

For a quantitative assessment of the ultrastructural effects of caffeine in rapidly frozen and in conventionally fixed EDL and SEMI muscles, we measured the volume of the TC in the three treatment groups (Table I): normal Ringer's solution, 2.0 mM caffeine, and 5.0 mM caffeine (contracture). The TC volumes measured with the digital planimeter (57) in conventionally fixed preparations were approximately: 1.8% (EDL) and 2.5% (SEMI) in control, 2.6% in 2 mM caffeine-treated EDL muscle (not shown in Table I), and 3.1% during 5 mM caffeine contracture in the SEMI muscle (all solutions contained 4% BSA). The increase in the TC volume in caffeine-treated muscles was significant ($P < 0.05$) and similar to the results published previously (80). The volumes obtained from point counting on the same material, at two spacings of 0.309 μm (× 7,000) and 0.175 μm (× 12,000) on calibrated micrographs (somewhat lower for each treatment group than measured with the planimeter), also showed swelling of the TC in caffeine-treated muscles. TC volumes in rapidly frozen and freeze-substituted preparations are also presented in Table I. Results (obtained with the planimeter) were 1.5% (EDL) and 2.1% (SEMI) in control, 1.5% in 2 mM caffeine-incubated EDL muscle (not shown in Table I), and 2.0% during caffeine contracture (SEMI), and show no significant effects of caffeine

FIGURE 3 Longitudinal section of semitendinosus muscle rapidly frozen during contracture with 5 mM caffeine and freeze-substituted. Disordering of sarcomere pattern and asymmetrical sarcomeres are indicated. Mitochondrial cristae and glycogen particles are clearly shown. TC with electron-opaque material (arrowheads) and T-tubule (arrows) can be seen. A, A band; I, I band; M, M line; Z, Z band; Mit, mitochondria. × 30,800; (inset) × 65,300.
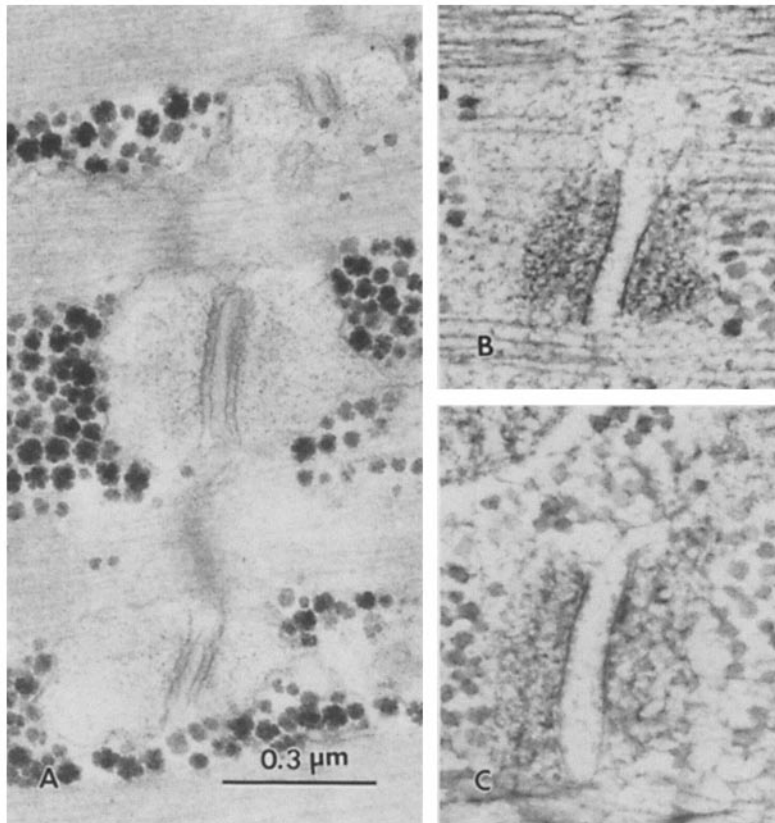
FIGURE 4 Longitudinal sections of a conventionally fixed muscle (A) and a rapidly frozen and freeze-substituted semitendinosus muscle (B and C) after 5 mM caffeine contracture for 1 min. Swollen TC can be seen in A, but not in B and C. × 66,900.

TABLE I

Volume Fraction of the Terminal Cisternae in Frog Skeletal Muscle

Condition	Muscle	Conventional fixation	Rapid freezing and freeze substitution
Digital planimetry			
Normal Ringer incubation	EDL	1.8 ± 0.45 (3, 245)	1.5 ± 0.33 (10, 1.865)
	SEMI	2.5 ± 0.81 (3, 357)	2.1 ± 0.71 (5, 850)
5 mM Caffeine contracture	SEMI	3.1 ± 1.21 (4, 602)	2.0 ± 0.81 (7, 418)
Point counting			
Normal Ringer incubation	EDL	1.3 ± 0.31 (10)	0.9 ± 0.22 (43)
	SEMI	2.1 ± 0.58 (9)	1.8 ± 0.49 (12)
5 mM Caffeine contracture	SEMI	2.9 ± 0.91 (20)	1.9 ± 0.85 (18)

Results are expressed as mean ± SD (percent of fiber volume). Each solution contained 4% BSA. The numbers in the parentheses for "digital planimetry" show the numbers of animals and measured TC, and for "point counting" the numbers of analyzed micrographs.

on the TC volumes. Similar results were obtained in all cases by using point-counting analysis (Table I).

In general, the TC volumes measured in each treatment group and by either method were smaller in the rapidly frozen and freeze-substituted than in the conventionally fixed muscles. However, the lateral (not adjacent to T-tubules) membranes of the TC were often ill-defined in freeze-substituted material, and in view of this and other considerations (see Discussion), we assessed the possible magnitude of the underestimate of the volume measurements. We remeasured a total of 69 TC (40 from caffeine contracture and 29 from resting muscles) with the planimeter, making one measurement by outlining the darkly stained area that we had included in all the tabulated measurements and a second measurement that included an indistinct, somewhat lucent, region adjacent to these TC. The volumes measured by including the electron-lucent regions in the measurements were 28 ± 2.0% SEM

greater (caffeine-treated) and 30 ± 2.9% greater (control muscles). Therefore, assuming a 30% underestimate of the measurements in freeze-substituted muscles, the TC volume in semitendinosus muscle would be ~2.7%.

Effects of Caffeine on Muscle Composition

The elemental composition representative of the whole fiber, measured with large (8–15 μm diam) probes in control and in caffeine-treated SEMI muscles, is shown in Table II. These analyses are representative of fiber regions including several sarcomeres and excluding mitochondria and nuclei. These results, most of which were obtained from paired (one caffeine-treated and one control) muscles, show that the elemental concentrations, with the exception of that of Cl, were not significantly affected by caffeine, and were within the normal range observed in frog muscle. The Ca content of the

TABLE II

Elemental Composition of the Whole Fiber at Rest and During Caffeine Contracture*

	n	Na	Mg	P	S	Cl	K	Ca
Rest	82	36 ± 26	43 ± 12	292 ± 64	195 ± 33	70 ± 21	442 ± 79	5.4 ± 3.4
1-min contracture	115	39 ± 17	40 ± 13	302 ± 41	202 ± 24	64 ± 22	462 ± 61	4.2 ± 3.7
Rest	18	41 ± 22	42 ± 12	303 ± 40	206 ± 21	84 ± 16	462 ± 58	2.2 ± 3.0
3-min contracture	35	45 ± 28	43 ± 10	294 ± 60	221 ± 29	60 ± 26	442 ± 96	3.9 ± 3.5
Rest (combined)	100	37 ± 25	43 ± 12	293 ± 60	197 ± 32	72 ± 21	445 ± 76	4.9 ± 3.5

* Mean ± SD mmol/kg dry weight.

muscles of the group of frogs (both control and caffeine-treated) used for determining the effects of caffeine after 3 min was rather low, possibly owing to seasonal variations. There was a statistically significant ($P < 0.001$) reduction in the total Cl content of the fibers analyzed with large-diameter probes after a 3-min caffeine contracture, but the sample size in this series was small. In cryosections of caffeine-treated muscle, the sarcomere pattern was frequently irregular, containing numerous regions of supercontracted and super-stretched sarcomeres throughout the fiber (Fig. 5).

Effect of Caffeine on the Composition of the TC of the SR

The results of the analyses of the TC with small-diameter (~50 nm) probes and of adjacent regions of the I band analyzed with identical probe parameters are summarized in Table III, and x-ray spectra of the TC and of the mitochondria of control and caffeine-treated muscles are shown in Fig. 6. These analyses were obtained from the same sections used for determining whole-fiber composition (Table II). To minimize the possible effects of frog-to-frog variations, we froze paired control muscles for the experiments in which the effects of 1- and 3-min-duration caffeine contractures were determined. The composition of the TC in resting muscle (Table III) was similar to that previously determined in frog muscle by electron probe analysis (43, 65), and since this has been described previously in detail, we shall only note here the changes in the contents of the TC due to the effects of caffeine (5 mM). The values given in mmol/kg dry wt can be converted to wet values by dividing them by 3.6, based on the ~72% H_2O content of the TC (65).

Caffeine caused a large and highly significant ($P < 0.001$) reduction in the Ca content of the TC from a resting value of ~115 mmol/kg dry TC weight to 40 mmol/kg dry TC weight within 1 min; the further reduction in the Ca content of the TC to 19 mmol/kg dry TC weight after 3-min exposure to caffeine was also significantly ($P < 0.001$) different than the value at 1 min, indicating the continued release of Ca between 1 and 3 min, albeit at a reduced rate.

The Mg content of the TC was significantly increased after 1 min of caffeine contracture, followed by a further small but significant ($P < 0.01$) increase at 3 min. Although the K content of the TC was also significantly ($P < 0.001$) increased after a 1-min caffeine contracture, by 3 min after initiation of the caffeine contracture it was not significantly different from that found in control, resting muscles.

The increase in Mg content of the TC (to 98 mmol/kg dry wt ± 17.9 SD; $n = 65$, four frogs) caused by a 5 mM caffeine (1 min) contracture was not reduced by pretreatment of the bundles with 5 μm valinomycin for 2 h at 2°C and, in fact,

was slightly greater than the value (88 ± 21) obtained without valinomycin. The sample size in this group of frogs was too small to determine the effect of valinomycin on the K content of the TC during caffeine contracture.

The total positive charge measurable in the TC by electron probe analysis (Na + K + 2Ca + 2Mg) was slightly (by 38 meq/kg dry wt with a 95% confidence limit of ± 34 meq/kg), but significantly ($P < 0.05$), reduced during a 1-min caffeine contracture. This result is not sufficiently different from the "apparent charge deficit" observed in tetanized muscle (60–80 meq/kg dry TC) and ascribed to charge compensation by proton flux into the TC (43, 65) to draw any definite conclusions. However, the continued Ca release accompanied by the absence of detectable K uptake after 3 min of caffeine contracture led to a very much larger apparent charge deficit compared with the resting values (117 meq/kg dry TC, $P < 0.001$). As discussed in detail previously (43, 65), most of the positive charge (cations) detected in the TC is bound to fixed charges on proteins within the TC lumen, and the apparent charge deficit between the charge released as Ca and that accumulated as $K^+ + Mg^{2+}$ is taken to reflect charge compensation by charges (protons and possibly organic ions) not detectable by electron probe analysis. Hence, the measured charge deficit is only apparent.

There was a significant increase in the P content of the TC and in the TC-cytoplasmic P gradient (at both 1 and 3 min), in contrast to observations on tetanized muscles in which there was no significant change in the P content of the TC (43, 65).

The concentration of Ca in the I band increased from 2.2 to 5.7 mmol/kg dry wt during a 1-min caffeine contracture and was slightly lower (4.2 mmol/kg dry wt, $P < 0.02$) after a 3-min caffeine contracture. The concentrations of most of the other elements (Na, P, S, and K) were higher in the I band during caffeine contracture than at rest and most probably represent increased hydration (and hence, an increased amount of elements in solution) in superstretched I bands. Chloride was the only element having a somewhat reduced concentration in the I band during caffeine contracture, paralleling the slightly lower values obtained in the measurements made with large-diameter probes (Table II).

Mitochondrial Ca Uptake

The composition of the mitochondria in control and in caffeine-treated muscles is summarized in Table IV, and a representative spectrum is shown in Fig. 6. The mitochondrial Ca content of caffeine-treated muscles was significantly increased. The Ca content of individual mitochondria was highly variable, as indicated by the large variance of these measurements. There was a tendency for mitochondrial Ca
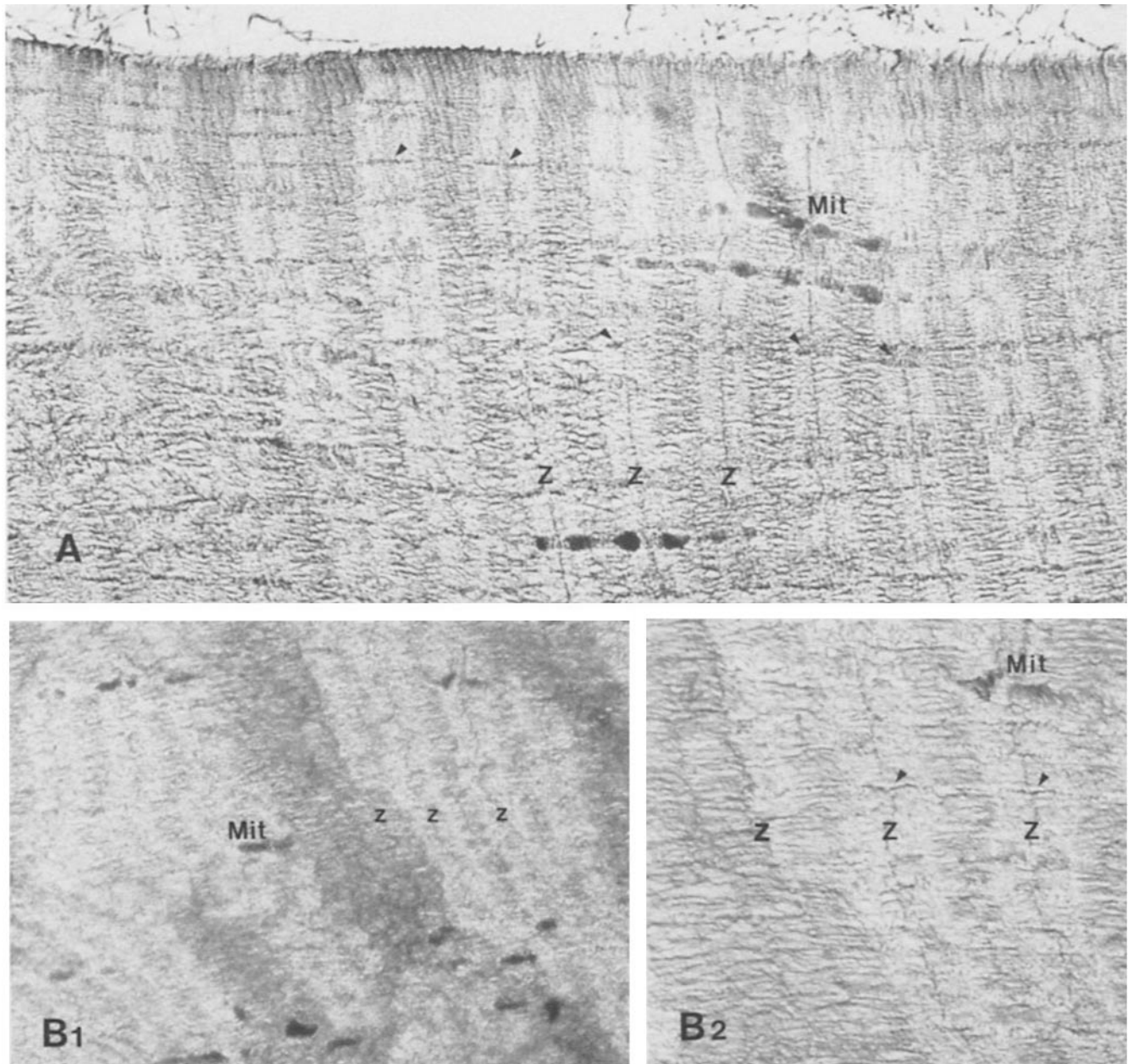
FIGURE 5 Longitudinal unstained cryosections of a control (A) and caffeine-treated (B1 and B2) semitendinosus muscles. Regular sarcomeres, Z lines, as well as TC (arrowheads) are apparent in A. In B1 and B2 (which are taken from the same fiber), the supercontracted sarcomeres can be seen. Z, Z band; Mit, mitochondria. (A and B2) × 7,400; (B1) × 3,700.

TABLE III

Effect of Caffeine on the Elemental Composition of the TC and the I Band*

	n	Na	Mg	P	S	Cl	K	Ca
TC								
Rest	111	45 ± 21	56 ± 12	472 ± 57	207 ± 30	58 ± 26	620 ± 74	113.5 ± 28.8
1-min contracture	134	57 ± 23	88 ± 21	565 ± 88	220 ± 42	53 ± 23	681 ± 131	39.6 ± 13.5
Rest	41	51 ± 30	60 ± 12	517 ± 83	236 ± 36	65 ± 14	628 ± 86	120.0 ± 35.5
3-min contracture	56	60 ± 27	97 ± 20	571 ± 89	231 ± 56	56 ± 23	639 ± 101	18.5 ± 10.5
Rest (combined)	152	46 ± 23	57 ± 12	482 ± 66	213 ± 34	60 ± 24	622 ± 77	114.9 ± 30.4
I band								
Rest	111	34 ± 24	52 ± 17	317 ± 84	213 ± 47	69 ± 23	460 ± 108	2.2 ± 4.4
1-min contracture	134	50 ± 27	58 ± 16	372 ± 89	242 ± 57	64 ± 26	521 ± 117	5.7 ± 3.9
Rest	41	38 ± 32	55 ± 13	350 ± 79	230 ± 41	79 ± 16	536 ± 88	3.2 ± 4.4
3-min contracture	56	40 ± 21	52 ± 11	363 ± 58	290 ± 32	57 ± 29	568 ± 86	4.2 ± 3.9
Rest (combined)	152	36 ± 26	53 ± 16	325 ± 84	217 ± 47	72 ± 22	480 ± 109	2.4 ± 4.4

* Mean ± SD mmol/kg dry weight.

content to be higher in the regions of supercontracted sarcomeres than in those of superstretched sarcomeres, but no attempt was made to quantitate this observation. The other elements measured by electron probe analysis in mitochondria were within the range of frog-to-frog variation found in this and previous studies (29, 43, 65, 66).

Pretreatment with valinomycin (5 μM for 2 h at 2°C) blocked the caffeine-induced increase in mitochondrial Ca. At the end of the 1-min caffeine contracture, the mitochondrial Ca in valinomcyin-treated muscles was 3.0 ± 3.2 SD (n = 37). That valinomycin penetrated (43) into these fibers was indicated by the marked increase in mitochondrial K (770 ± 183.4 SD).

DISCUSSION

Ca Release, Mg Uptake, and Other Changes in TC Composition during Caffeine Contracture

The marked reduction in the Ca content of the TC from control values of ~115 mmol/kg dry TC to, respectively, 40 and 19 mmol/kg dry TC at 1 and 3 min after the initiation of caffeine contracture confirms the earlier conclusions (20, 75, 76) that caffeine contractures are due to the net release of Ca from the TC (heavy SR) of the SR. More Ca was released in situ (present study) already at 1 min than the maximal release obtained from fragmented SR preparations (39, 49, 53, 75, 76), and release became even greater (~100 mmol/kg dry TC) after 3 min of contracture. The greater Ca-releasing effect of caffeine in situ than in vitro is even more noticeable if we consider that the present experiments were conducted
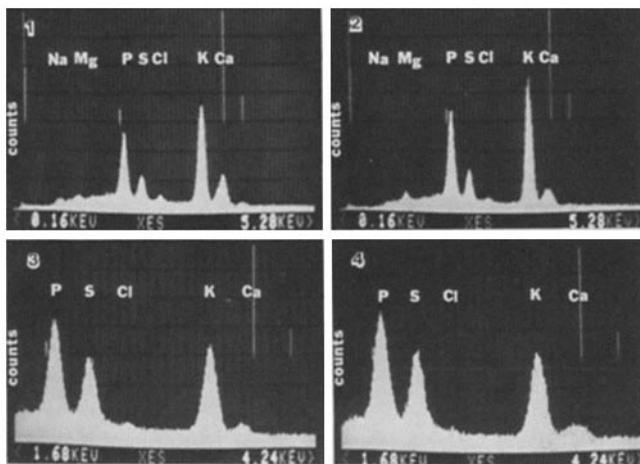

FIGURE 6   X-ray spectra of the terminal cisternae (1 and 2) and mitochondria (3 and 4) of a control (1 and 3) and a caffeine-treated (2 and 4) muscle. High concentration of Mg and low Ca was found after a 5-mM caffeine contracture in the TC (2), and Ca was increased in mitochondria (4).

at room temperature, at which the Ca-releasing action of caffeine on fragmented SR (53, 76) and in situ (58, 59) is less than at lower temperatures. A further difference between the Ca-releasing effect of caffeine on muscle and on fragmented SR is the fact that in isolated SR preparations Ca release is transient: reuptake occurs in the presence of caffeine (49, 53), whereas in intact muscle it appears to be sustained for at least 3 min. After 3 min of a contracture evoked by 5 mM caffeine, ~84% of the total Ca content of the TC had been released. This observation is consistent with experiments on skinned muscle fibers that showed the complete release of Ca by 25 mM caffeine (20, 22, 23, 68, 69). The amount of Ca released during a 1-min caffeine contracture was similar to the magnitude of release during a 1.2-s tetanus (43, 65), and it became very significantly greater at 3 min.

A highly significant uptake of Mg into the TC also accompanied caffeine contractures (Table III). Like the release of Ca, the uptake of Mg was also significantly greater at 3 min than at 1 min after the initiation of the caffeine contracture. An uptake of Mg into the TC is also associated with Ca release during tetanus, and it has been suggested that this may be due to passive influx of Mg into the TC as partial neutralization of the electrical charge released in the form of Ca^{2+}, with Mg possibly using the same channels as Ca efflux (65). The increase in the Mg content of the TC caused by caffeine (present study) is also compatible with Mg movement through common, caffeine-sensitive Ca-Mg channels. If so, then these findings lend further support to the above hypothesis for the following reasons: as caffeine increases the passive permeability of the SR to Ca^{2+} (40), it would be expected that the movement of another ion (i.e., Mg) through the same channels would inhibit Ca^{2+} movement. This in fact appears to be the case, as the release of Ca from fragmented SR is increased at low Mg and inhibited by high Mg (24, 75; but cf. reference 70), and passive Ca influx due to caffeine in skinned fibers is also inhibited by high Mg (40). That the extent of Mg uptake into the TC was proportional to the amount of Ca release (approximately 0.4 Mg/Ca) rather than to the duration of exposure to caffeine (i.e., greater during the first minute of the caffeine contracture than during the next 2 min) also supports the interpretation that these Mg movements are not secondary to Ca-ATPase activity. That valinomycin, a K ionophore that increases K uptake into the TC in a tetanized muscle (43) does not reduce the Mg uptake into the TC during caffeine contracture (present study) or during tetanus (43) suggests the presence of a rather high permeability and/or strong driving force on Mg that is not readily short-circuited by K.

The uptake of K into the TC during a 1-min caffeine contracture was significant and comparable to that occurring during a 1.2-s tetanus (43, 65), and the reduction in the total amount of positive charge (Na + K + 2Ca + 2Mg) measurable by electron probe analysis in the TC was either similarly or

less reduced. However, after a 3-min caffeine contracture, the K content of the TC was not significantly different from that in control (resting) muscles, despite the further reduction in Ca, leading to a very marked apparent charge deficit in the TC. We can only speculate that this large apparent charge deficit, after a 3-min caffeine contracture, may represent acidification of the cytoplasm and proton movement into the SR during this period and/or the dispersion, with change in the binding properties of the cation-binding proteins normally concentrated in the TC (e.g., calsequestrin, references 35 and 37), to other regions of the SR. The latter possibility is suggested by the change in physical state of calsequestrin caused by low Ca in vitro (33). However, dispersion of calsequestrin alone would not account for the lack of K uptake, as Mg (also thought to be bound to calsequestrin) was further increased during this time period.

The increased P content of the TC due to caffeine contracture (at both 1 and 3 min) may be related to changes in cytoplasmic organic phosphorus compounds. We are not aware of ^{31}P nuclear magnetic resonance data obtained during similarly short caffeine contractures, but prolonged caffeine contractures cause a marked increase in the orthophosphate, and reduction in creatine phosphate and ATP, content of frog muscle (reference 9 and M. Bárány, personal communication).

Mitochondrial Ca Uptake and Fiber Ca Concentration

In contrast to the complete lack of mitochondrial Ca accumulation in normal, tetanized (65) or fatigued (29) frog muscle, in normally contracting smooth muscle (61–63), and in cyclically contracting isolated heart cells (12), significant Ca accumulation (11–16 mmol/kg dry mitochondrial weight) was observed after a caffeine contracture (present study). The large variance of mitochondrial Ca content in caffeine-treated muscles (Table IV) reflected the individual variability of mitochondrial Ca concentrations, and was very much greater than the statistical errors of the measurements (compare standard deviations of the control with caffeine-treated values in Table IV). There are two plausible reasons for the observed mitochondrial uptake of Ca during caffeine contracture, as compared with the absence of such uptake in tetanized frog muscle and in fact in all normal cell systems studied to date (63). Free Ca^{2+} during caffeine contracture may have risen above the levels reached during normal tetanus, and/or the much longer duration of the caffeine contracture (than tetanus) may have allowed mitochondria to accumulate Ca even in the presence of very modest rates of mitochondrial Ca uptake. The free Ca concentration during maintained caffeine contracture in frog muscle is not known, as far as we can ascertain. In our experiments, Ca release during a 1-min caffeine contracture (~65% of total) was similar to the amount released (~60–70%) during tetanus, but even a small increment in the total Ca released would cause a large increase in free cytoplasmic Ca^{2+}, if it exceeded the capacity of the cytoplasmic Ca-binding sites (e.g., troponin and parvalbumin). Furthermore, the small uptake (11 mmol/kg dry mitochondrial weight during the first minute) and long duration of the caffeine contracture require very modest rates of Ca transport (0.3–0.4 nmol Ca/mg mitochondrial protein·s⁻¹). Net uptake at such low rates for only 1.2 s would not be detectable with the probe parameters used for electron probe analysis.

The increase in mitochondrial Ca observed in situ (present study) is consistent with earlier findings showing that caffeine causes a shift of Ca from the microsomal to the mitochondrial fraction (7), and the lack of inhibitory effect of caffeine on isolated mitochondria (75). The only slight (5 mmol/kg) additional mitochondrial Ca uptake after 3 min suggests that Ca^{2+} may have declined after 1 min, because mitochondria can accumulate much higher concentrations of Ca (up to 2 mol/kg) when exposed to high free Ca^{2+} (61–63). The inhibition of mitochondrial Ca uptake by valinomycin is consistent with the uncoupling action of this K ionophore.

The cytoplasmic Ca concentration measured with small probes in the I band (5.7 mmol/kg dry wt) was significantly increased during caffeine contracture, while the total Ca content in the fiber (averaged with large-diameter probes) was unchanged (Table III). The unchanged Ca content of the fiber after a 3-min caffeine contracture is consistent with the observed release (together with influx) of ⁴⁵Ca from frog muscle treated with caffeine (5, 7).

Mechanism of Caffeine Action

Both the amount (19–21) and duration (present study) of Ca release caused by caffeine is significantly greater in situ than in isolated SR preparations. These findings are consistent with, though do not prove the interpretation, that caffeine acts through the release of some second messenger than can accumulate to a detectable concentration in whole fibers (45), but is readily exhausted or inactivated in fragmented SR preparations. Since the Ca-releasing action of caffeine is itself dependent on Ca^{2+} (19, 75), one may speculate that its action involves stimulation of a Ca-dependent enzyme located in the T-tubule-SR region. Our finding that caffeine, like tetanic stimulation, also increases the uptake of Mg into the SR is more consistent with the notion that caffeine increases the passive permeability of the SR to Ca (19) than with an inhibition of Ca uptake as the major mechanism (e.g., reference 51). This interpretation is consistent with observations showing that the Ca-ATPase is inhibited by caffeine only at very low (<0.1 mM) ATP concentrations (75), very much below those occurring in vivo, and does not influence the level of phosphorylated enzyme intermediate (54) or slow significantly the rate of relaxation of a tetanus (47) or a K contracture (10) superimposed on caffeine contractures. Unfortunately, neither the measurement of the total Ca remaining in the SR after caffeine treatment, whether through force responses to caffeine (19–21), with ⁴⁵Ca (69), or by electron probe analysis (present study), nor observations on the decline of free Ca^{2+} with Ca-sensitive indicators (3, 4, 44) can unambiguously distinguish an increased Ca leak from inhibition of uptake (or a combination of the two), as both mechanisms cause the same net effect (reduction of the Ca content of the TC and increase in the cytoplasmic Ca^{2+}) in the experimentally measurable quantities.

The two major structural observations made in this study were that the SR was not swollen during caffeine contracture in cryopreserved muscles and that, in general, the volume of the TC is smaller in rapidly frozen and freeze-substituted than in conventionally fixed material. Swelling of the TC as the result of glutaraldehyde-osmium fixation has also been observed in muscles pretreated with hypertonic solutions (6, 32) that in rapidly frozen and freeze-substituted preparations were shown to contain unswollen SR and vacuolated T-tubules (28, 66). Prefixation with aldehydes does not abolish the

osmotic response of cells, and either swollen or shrunken appearance of cells and organelles can be obtained in aldehyde-osmium–fixed material by modifying the tonicity of the buffer used during fixation (13, 18, 32, 34). The preservation of valinomycin-induced swelling of mitochondria in rapidly frozen and freeze-substituted frog muscle (43) and the absence of apparent osmotic driving force (see below) argue against the possibility that cryopreservation caused a reversal of swelling present in vivo.

The absence of swelling of the SR during caffeine contracture implies that rupture of the SR membranes due to gross distension is *not* the mechanism of caffeine-induced Ca-release, and this same conclusion can also be reached from the reversibility of caffeine-induced Ca-release in skinned muscles (19) and fragmented SR preparations (20, 75). The absence of a significant electrical or ionic gradient across the SR membrane in resting muscle (43, 65, 66; for review see references 30 and 52) also indicates the absence of a driving force that could lead to solute movements and accompanying water flow into the SR under the influence of caffeine. Indeed, we found no net increase in the concentration of osmotically active components detectable by electron probe analysis in the TC during caffeine contracture (see below). In comparing the present results with earlier studies showing caffeine-induced swelling of the SR in aldehyde-fixed material (15, 31, 72), we note that some of these were performed after prolonged caffeine contracture (e.g., 20–30 min, 10 mM caffeine, see reference 31), and were associated with marked distortion and expansion of the interfibrillar space. The latter changes may have been due to the loss of soluble cytoplasmic proteins during the prolonged rise in cytoplasmic free Ca^{2+} caused by caffeine (71). Other than the effects of mechanical distortion due to loss of proteins and regions of supercontraction (16, 36, 55), caffeine-induced swelling of the SR in aldehyde-fixed preparations could also be caused by a permeability change in the SR membrane or depolymerization of intraluminal proteins (e.g., calsequestrin) that might predispose to swelling during chemical fixation. In any event, neither of these mechanisms appears to be operating in living muscle, as judged by the electron microscopic appearance of rapidly frozen preparations.

The fractional volume of the TC measured was smaller in the rapidly frozen and freeze-substituted than in the conventionally fixed preparations, and our values for the latter were somewhat smaller (2.5 vs. 3.5%) than those obtained (in two semitendinosus muscles) by Mobley and Eisenberg (50). We shall not be concerned with the latter discrepancy, which could be due to either biological variation or the different fixative buffers used in the two studies, as the variations in the appearance of the SR in conventionally fixed muscles are well-known to electron microscopists. However, it is necessary to consider whether the volume of the TC in vivo is closer to that measured after freeze substitution or after conventional fixation. Inasmuch as the stereological measurements are expressed as fractional volumes (TC volume as percent of fiber volume), the degree of linear shrinkage estimated in this and other (8) studies (11–20%) during freeze substitution should not affect our measurements, unless the volume changes due to processing are different in the TC than in the filamentous proteins. We were led to consider this possibility by a discrepancy between the rise of the Ca content measured in the I band using caffeine contracture and the amount of Ca released from the TC (see below) and the observation

(pointed out to us by Dr. A. V. Somlyo) that an electron-lucent halo is often, though not invariably, observed around TC and mitochondria in freeze-substituted muscles (see, for example, Fig. 3 in reference 65; present study). For example, an increase in the Ca concentration (3.1 mmol Ca/kg dry myofibril) into a fibrillar volume (excluding SR and mitochondria) of 88%, if due to the decrease in the Ca content of the TC of 74 mmol/kg dry TC (Table III), would require a TC volume of ~3.6%. Given the statistical errors of the measurements involved, there is only about a 5% possibility that the increase in cytoplasmic Ca measured could originate from a TC volume that constituted 2.2–2.7% of the fiber. Furthermore, such a small increase (~1.9 mmol/kg dry wt) in the Ca content of the I band during caffeine contracture is inconsistent with the concentration of high-affinity Ca binding sites (troponin and parvalbumin) in this region (3, 65). It is also possible that the less intense staining of the membranes in the freeze-substituted than in the conventionally fixed muscles could obscure a larger proportion of tangentially sectioned membranes (46, 56). Therefore, we consider it possible, and even likely, that the absolute fractional volumes of the TC measured in freeze-substituted preparations may represent an underestimate of the in vivo value in normal muscles. Somewhat surprisingly, the estimate of the volume of the TC in conventionally fixed, normal muscles could be closer to the in vivo values than the volumes found in rapidly frozen preparations.

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