Calcium Release and Ionic Changes in the Sarcoplasmic Reticulum of Tetanized Muscle: An Electron-Probe Study

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ABSTRACT Approximately 60–70% of the total fiber calcium was localized in the terminal cisternae (TC) in resting frog muscle as determined by electron-probe analysis of ultrathin cryosections. During a 1.2 s tetanus, 59% (69 mmol/kg dry TC) of the calcium content of the TC was released, enough to raise total cytoplasmic calcium concentration by ~1 mM. This is equivalent to the concentration of binding sites on the calcium-binding proteins (troponin and parvalbumin) in frog muscle. Calcium release was associated with a significant uptake of magnesium and potassium into the TC, but the amount of calcium released exceeded the total measured cation accumulation by 62 mEq/kg dry weight. It is suggested that most of the charge deficit is apparent, and charge compensation is achieved by movement of protons into the sarcoplasmic reticulum (SR) and/or by the movement of organic co- or counterions not measured by energy dispersive electron-probe analysis. There was no significant change in the sodium or chlorine content of the TC during tetanus. The unchanged distribution of a permeant anion, chloride, argues against the existence of a large and sustained transSR potential during tetanus, if the chloride permeability of the *in situ* SR is as high as suggested by measurements on fractionated SR.

The calcium content of the longitudinal SR (LSR) during tetanus did not show the LSR to be a major site of calcium storage and delayed return to the TC. The potassium concentration in the LSR was not significantly different from the adjacent cytoplasmic concentration. Analysis of small areas of I-band and large areas, including several sarcomeres, suggested that chloride is anisotropically distributed, with some of it probably bound to myosin. In contrast, the distribution of potassium in the fiber cytoplasm followed the water distribution. The mitochondrial concentration of calcium was low and did not change significantly during a tetanus. The TC of both tetanized and resting freeze-substituted muscles contained electron-lucent circular areas. The appearance of the TC showed no evidence of major volume changes during tetanus, in agreement with the estimates of unchanged (~72%) water content of the TC obtained with electron-probe analysis.

The release of Ca from and its subsequent return to the triadic portion of the sarcoplasmic reticulum (SR) (28, 80, 82) are the major determinants of the contractile cycle of striated muscle (for review, see reference 24). Since the demonstration of the SR as the ATP-dependent relaxing factor (57), a wealth of information has been accumulated about the kinetics and mechanisms of calcium uptake by the SR (e.g., 41, 48, 63, 104, 106, and for review, see references 62, 102). In contrast, comparatively little is known about the mechanism of release and associated ion movements, largely because isolated SR preparations do not lend themselves to reproduction of the physiological release process nor maintain their monovalent ion

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composition during isolation. Thus, until recently, the question of whether the SR of resting muscle is a truly intracellular compartment or one in ionic communication with and similar in ionic contents to the extracellular space was the subject of some debate (for review, see references 74, 96). The availability of electron-probe microanalysis at high spatial resolution combined with rapid freezing techniques to preserve the in vivo distribution of diffusible elements (for review, see references 39, 44, 59, 60, 93) made it possible to measure the inorganic content of cell organelles *in situ*. The application of these methods to striated muscle established that the terminal cisternae (TC) of the SR have a monovalent ion composition different from that of the extracellular space and also corroborated the well established notion (21, 66, 78, 81, 108, 109) that they are the major site of Ca sequestration in resting muscle (96, 97). The feasibility of freezing at different stages of the contractile cycle permits the further extension of this approach to determining changes in the composition of the SR during various phases of activation and relaxation. Some of the major questions that remained unresolved about contractile activation have been the total amount of Ca released, the counter-and/or coions that are moving into or out of the SR during Ca release and the changes in ionic equilibria and electrical potential across the SR. The purpose of our study was to obtain some answers to these questions through electron-probe analysis of muscles rapidly frozen during tetanus. It is anticipated that future studies will deal with the changes during relaxation and twitch. Some of our preliminary results have been presented at the Kumagai-Natori (90) and the Jerry Lewis Symposia (95) and in preliminary communications (94).

MATERIALS AND METHODS

Bundles of 20–25 fibers with both tendons attached were dissected from both dorsal frog semitendinous muscles from ten *Rana pipiens*. One muscle from each animal served as the control resting sample and the other as its tetanized pair. The dissection was done at room temperature and the fibers were allowed to rest for 30 min to 2 h at 4°C. Before use, the muscles were checked under the dissecting microscope for their ability to twitch when stimulated and for the presence of damaged (opaque) fibers. Damaged preparations were discarded. Bovine serum albumin 4 g/100 ml (#6003 fatty acid free, Sigma Chemical Co., St. Louis, Mo.) was added to the frog Ringer's solution, pH 7.2, 10 min. before freezing, to minimize ice-crystal formation in the extracellular space. Muscle bundles were mounted on specially designed low mass stainless steel mesh holders and stretched to ~2.8 μm sarcomere length. The domed portion of the holder was covered with a very thin sheet of frog mesentery to reduce damage to the muscle fibers next to the holder. Force was recorded with an RCA 5734 transducer. An agar electrode positioned across the surface of the bundle served as one electrode and the holder served as the other. Trial shocks of increasing voltage were given to determine maximal twitch size. The twitch/tetanus ratio was 0.5 ± 0.16 SD (n = 20). Muscles were kept moist with drops of Ringer's solution. One member of each muscle pair was tetanized with 40 (5 ms duration) shocks/s. At 1.2 s, a 200-ml beaker of supercooled Freon 22 (93, 96) was shot up to the muscle at 80–100 cm/s. Tension was monitored throughout (Fig. 1). The paired control muscle was treated in a similar fashion but was not tetanized. Sections 1,000–2,000 Å thick (usually gold, occasionally purple interference colors) were cut on a LKB cryoultramicrotome (LKB Productor, Bromma, Sweden), modified to maintain an ambient temperature of −130°C in the cryochamber, a specimen temperature of −110°C and glass knife temperature of −100°C as published in detail previously (93, 96). Sections were removed with chilled orange stick splinters from the knife and placed on thin carbon foils on Cu grids, glow discharged to increase the adhesiveness of the sections. Transfer to the vacuum evaporator (Denton Vacuum Inc., Cherry Hill, N. J.), and drying at or below 5×10^{-6} Torr followed by carbon coating were identical to the procedures used in our previous studies of striated and smooth muscle (89, 96). All the tabulated electron-probe data were obtained on unstained freeze-dried cryosections. After cryosections were obtained, the remaining portions of the muscles were processed by freeze substitution (for review, see references 10, 29) for morphological studies.

Electron-probe analysis was done in a Philips EM400 transmission electron microscope fitted with a goniometer stage, field emission gun, scanning transmission electron microscope (STEM) attachment and a 30 mm^2 Kevex Si(Li) energy dispersive x-ray detector (Kevex Corp., Foster City, Calif.) and Kevex model 7000 multichannel analyzer interfaced to a PDP 11/34 computer system (Digital Equipment Corp., Marlboro, Mass.). For the x-ray map shown in Fig. 6, a 30 mm^2, 18° take-off angle Edax detector (Edax International Inc., Philips Electronic Instruments, Inc., Prairie View, Ill.) was used. The equipment configuration and its operating characteristics have been described elsewhere (89). Information from the electron and x-ray detector systems was collected on-line and stored on magnetic disc until required for processing. The microscope was operated at an accelerating voltage of 80 kV and a probe current of ~1 nA. All analyses were done with a liquid N_2-cooled holder at −165°C, to minimize contamination and radiation damage (87).

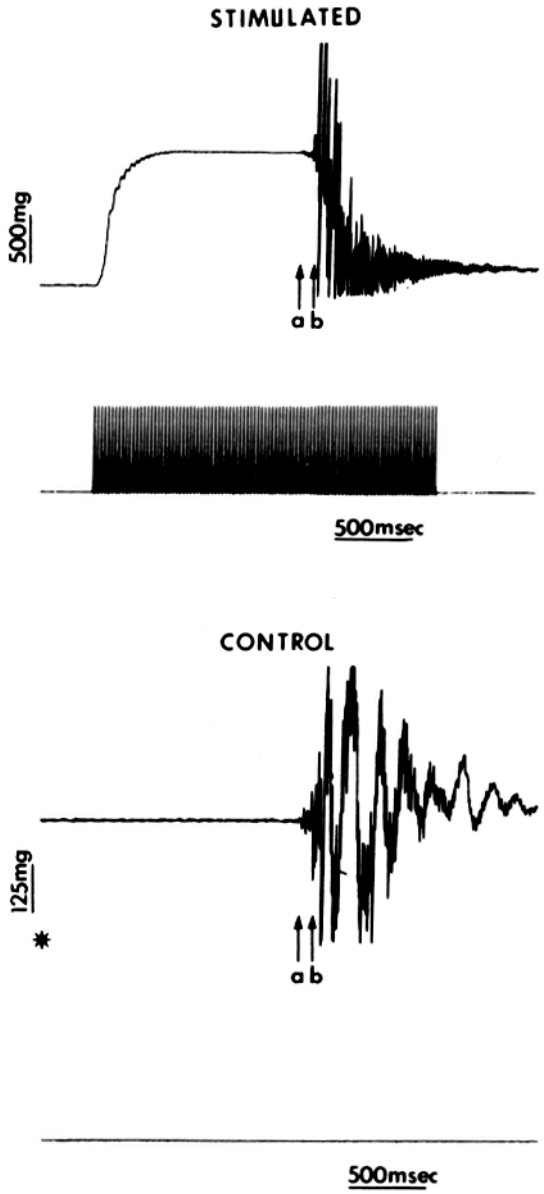

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FIGURE 1 Tension traces recorded before freezing a tetanized and a control frog semitendinous muscle bundle of 15–20 fibers. Muscles were first stimulated to twitch at increasing voltage to determine the voltage for activation of all fibers. A tetanus was produced by stimulating the muscle for 1.2 s at a rate of 40 supermaximal shocks/s. At 1.2 s, a beaker of supercooled Freon was shot up to the muscle (point *a*) and contact was made at point *b*. The noise in the tension record is due to the beaker of Freon traveling at 100 cm/s and the transducer touching the side of the beaker in this experiment.

The x-ray spectrum obtained in a transmission electron optical column from a thin section of biological material includes the characteristic peaks due to elements with atomic numbers >10 present in the specimen and the associated continuum largely arising from the organic matrix. The spectrum also contains instrumental peaks (the largest one being due to copper at 8.04 keV) from the specimen grid and holder, with an associated extraneous continuum, and low energy noise probably due to electrons reaching the detector. The extraneous signals can be determined and subtracted from the spectrum (87, 88). The method used to convert x-ray spectra to concentrations is based on the fact that the ratio of the characteristic peak counts to the counts in the x-ray continuum generated by the specimen is proportional to elemental concentration (38); this has been described and validated in detail (87). The statistical analyses (5) were also described in our previous publications (87, 89, 96). Continuum counts were measured in the energy region of 1.34 keV, and are directly proportional to specimen mass (related to section thickness) and to the probe currents used. Therefore, direct comparisons of these counts should only be made between paired analyses performed with the same probe parameters within two adjacent regions of the same section (e.g., of continuum ratios between paired TC and cytoplasm). Negative concentrations are due to statistical fluctuations and have no biological significance. The instrumental error of the mean (IEM) was small, ≤0.3 mmol/kg dry wt in the data shown in Tables III and V, where

$$\text{IEM} = \sqrt{\frac{1}{\Sigma\left(\frac{1}{\sigma_i^2}\right)}}$$

and σ_i is the uncertainty of the individual measurements. The IEM is dominated by the Poisson statistics of x-ray counting and, as the number of measurements increases, the IEM decreases. Hence, the statistical uncertainties of our results are largely due to biological variations.

Estimates of errors of measurement and of cell-to-cell variability (89) and the results of quantitative analysis of standards (87) have been published. The measurement of low (i.e., cytoplasmic) Ca concentrations is subject to three main sources of error, one of which (changes in detector calibration) has been considered previously (87, 89). Two other sources of error are low level Ca contamination of the carbon support films and the possible inclusion of tangentially sectioned and, therefore, invisible regions of SR in the probed region of I-band cytoplasm. Each of these errors will increase the measured "cytoplasmic" Ca concentration. To assess the magnitude and minimize the source of these artifacts, carbon films supporting an analyzed cryosection were analyzed for each of the ten muscles used with the same probe parameters as the cytoplasm (large probe measurements) of one of the fibers. The Ca counts obtained from the carbon film compared to the Ca counts from the "large cytoplasmic" analysis give a measure of the possible overestimate in Ca concentrations due to contamination of unselected carbon films. In ten paired carbon-film and cytoplasmic measurements, the overestimate due to contamination found in this manner was 0.4 mmol/kg. Transverse cryosections, in which the collar of SR around the I-band can be avoided, were also obtained from other resting muscles and placed on carbon films that were previously analyzed and ascertained to be free of Ca. Finally, the spectra obtained in these analyses were stripped by the computer program; this procedure removes the characteristic peaks leaving the x-ray continuum to be displayed on the multichannel analyzer. In this manner, the goodness of fit in the K + Ca region can be determined by visual assessment, and spectra showing a shift in detector calibration (centroid or resolution) can be excluded. The results of each of these procedures and the more conventional means of excluding rogue values (more than three SD above mean value) were used to assess the Ca concentration in the cytoplasm of resting muscles. In tetanized muscles, the effect of including small regions of nonvisualized SR in cytoplasmic analysis is reduced, due to the lower Ca concentration of the SR in stimulated muscles (see Results).

X-ray maps for particular elements were generated by using the STEM attachment to produce a raster scan and recording, at each picture point, the number of x-ray counts occurring in a narrow window centered on the energy characteristic for the element (91). The x-ray maps and simultaneously recorded STEM images were built up from 512 lines (generally 1 s/line), each containing 512 picture elements (i.e., a total of 262,144 picture elements). Up to 4 maps and one image could be collected simultaneously, and repeated frames added to improve count statistics. Grey scale images were generated on a video screen using a DeAnza VC5000 image display system (DeAnza Corp., San Jose, Calif.) which enables 16 bits of information to be stored for an image of up to 512 × 512 picture elements. The images shown in Figs. 6 and 8 are photographs taken directly off the screen of this device.

Approximately 500 Å diameter probes were used for spot mode analysis of individual TC and adjacent cytoplasm. Given our thin cryosections and the orientation of the TC around the myofibrils (~0.5 μm in diameter), it is possible to obtain transversely sectioned TC which run through the entire depth of these longitudinal sections. Great care was taken in selecting discreet oval pairs of TC where frequently a small transverse tubule could be seen between them (figs. 4 and 8).

Because of the domed shape of the specimens, the peripheral portions of the sections were well frozen with no ice crystals visible at × 20,000. Imaging of organelles and sarcomeres was difficult in these regions due to the low contrast. Contrast was markedly improved in areas where small ice crystals occurred, such as in fig. 4. The majority of the analyses were carried out on this type of specimen. The probe diameters used were always several times larger than the size of the ice crystals. Analysis of both slightly icy and ice crystal-free regions within the same fiber gave similar results.

RESULTS

Structure

In both the cryosections and the plastic sections of freeze-substituted material, the depth of tissue which showed no evidence of ice-crystal damage at × 20,000 ranged from 2 to 10 μm from the surface (Figs. 2, 3, 4, and 5). The quality of freezing achieved (see Materials and Methods) is best illustrated in Fig. 2, in which the morphology is well preserved. This muscle, which was flash-frozen during the rising phase of tetanic tension when the force had reached 50% of maximum, shows slight disordering of the myofibrils and the occasional slippage of (out of register) myosin filaments. As the cryosections were cut from a slightly domed surface, the first few sections as well as the outer aspect of all subsequent sections were well-frozen. In sections taken from the deeper portions of the specimen, ice-crystal size increased toward the center. This effect can be seen when comparing the right and left portions of the cryosection in Fig. 4. Myosin filaments can be resolved in the better-frozen portion of this section (see *inset*, Fig. 4). The contrast variations seen in the unstained frozen-dried cryosections arise from the differences in mass.

The 1,000–2,000 Å thickness of cryosections, in conjunction with tiny compression lines or ridges resulting from fracturing of the section, prevented the resolution of the details of the triadiac gaps (22, 28, 92), although discreet TC could be imaged for analysis (Fig. 4). No change was observed in the freezing properties of the TC of the tetanized as compared with the resting muscles. The absence of any visible change in the propensity for ice-crystal damage in the SR in the cryosections as well as no apparent volume change in the thin sections of freeze-substituted fibers, suggest that there is no substantial swelling or shrinkage of this membrane system during a tetanus.

Thin sections of well-frozen freeze-substituted plastic embedded, stained material allowed better resolution of the triads. Periodic, circular electron-lucent areas within the TC facing the junctional gap (Figs. 2 and 3) were consistently observed in all of the resting and tetanized muscles examined by freeze-substitution. Similar structures have been observed in deep etched muscles (75).

Electron-Probe Analysis

FIBER, A-, AND I-BAND COMPOSITION: The results of analysis using large probe diameters (6–20 μm) averaging over several sarcomeres of control and tetanized fibers are shown in Table I. The elemental concentrations are expressed on a dry weight basis. Given a 77% H_2O content of frog muscle fibers, the 427 mmol K/kg dry wt is equivalent to 129 mmol K/l fiber H_2O, in excellent agreement with values obtained with chemical analyses as well as K sensitive electrodes (for review, see reference 96). The Na, Mg, P, S, Cl, K, and Ca concentrations were not significantly different in the 49 control and 43 tetanized fibers from the paired muscles of ten frogs.

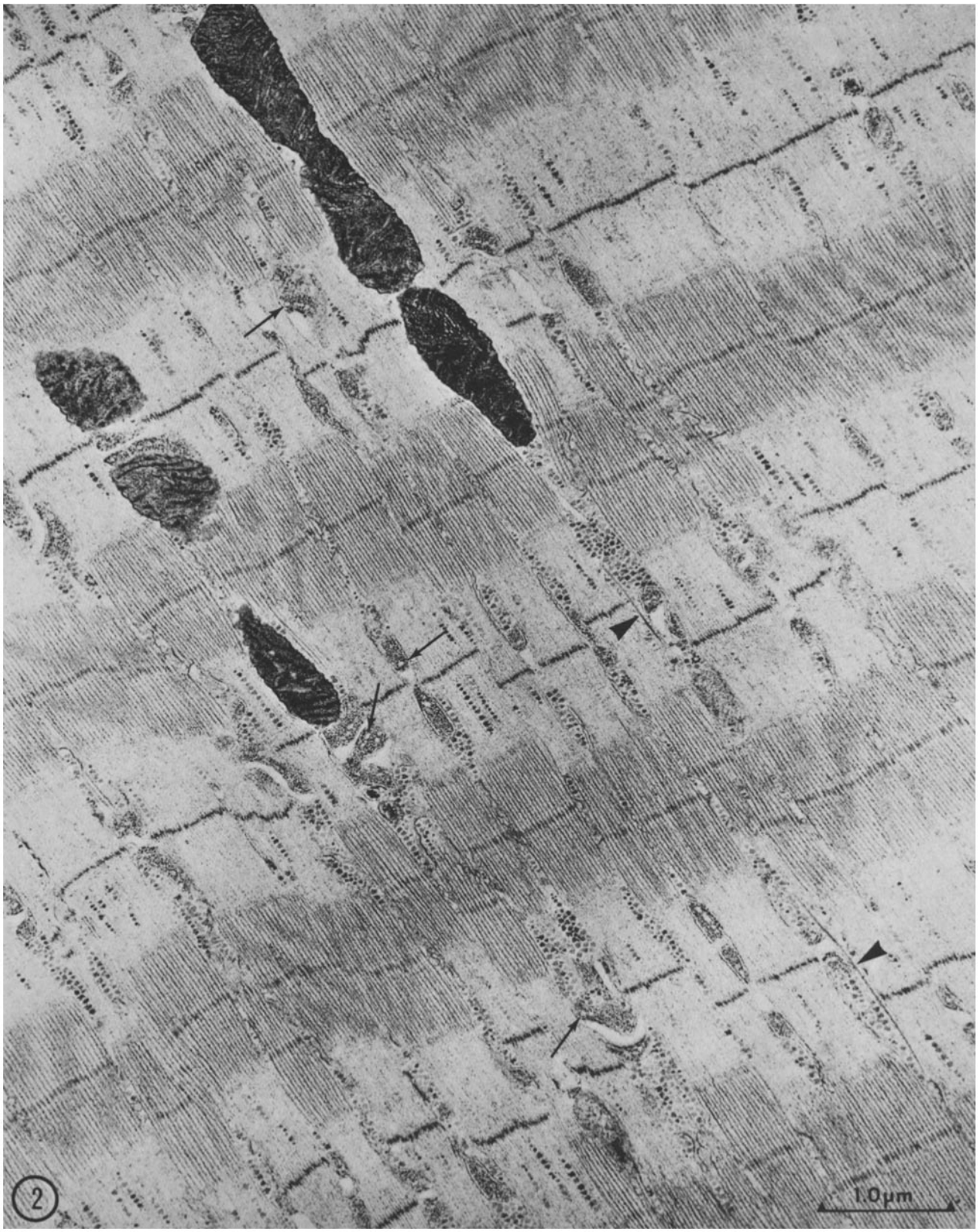


FIGURE 2 Longitudinal section from the outer portion of a muscle frozen during tetanic tension development and processed by freeze-substitution. Tension had reached 50% of full tetanic tension. Ice crystal damage is not evident. The volume of the SR and the densely staining material in the TC do not appear different from the resting fibers frozen by the same techniques. The triads are somewhat misaligned in relation to the Z-line in this long sarcomere fiber. Occasional myosin filaments (arrowheads) appear to have slipped into the I-band. Periodic electron-lucent circular areas are in the TC facing the junctional gap (arrows). × 24,000.

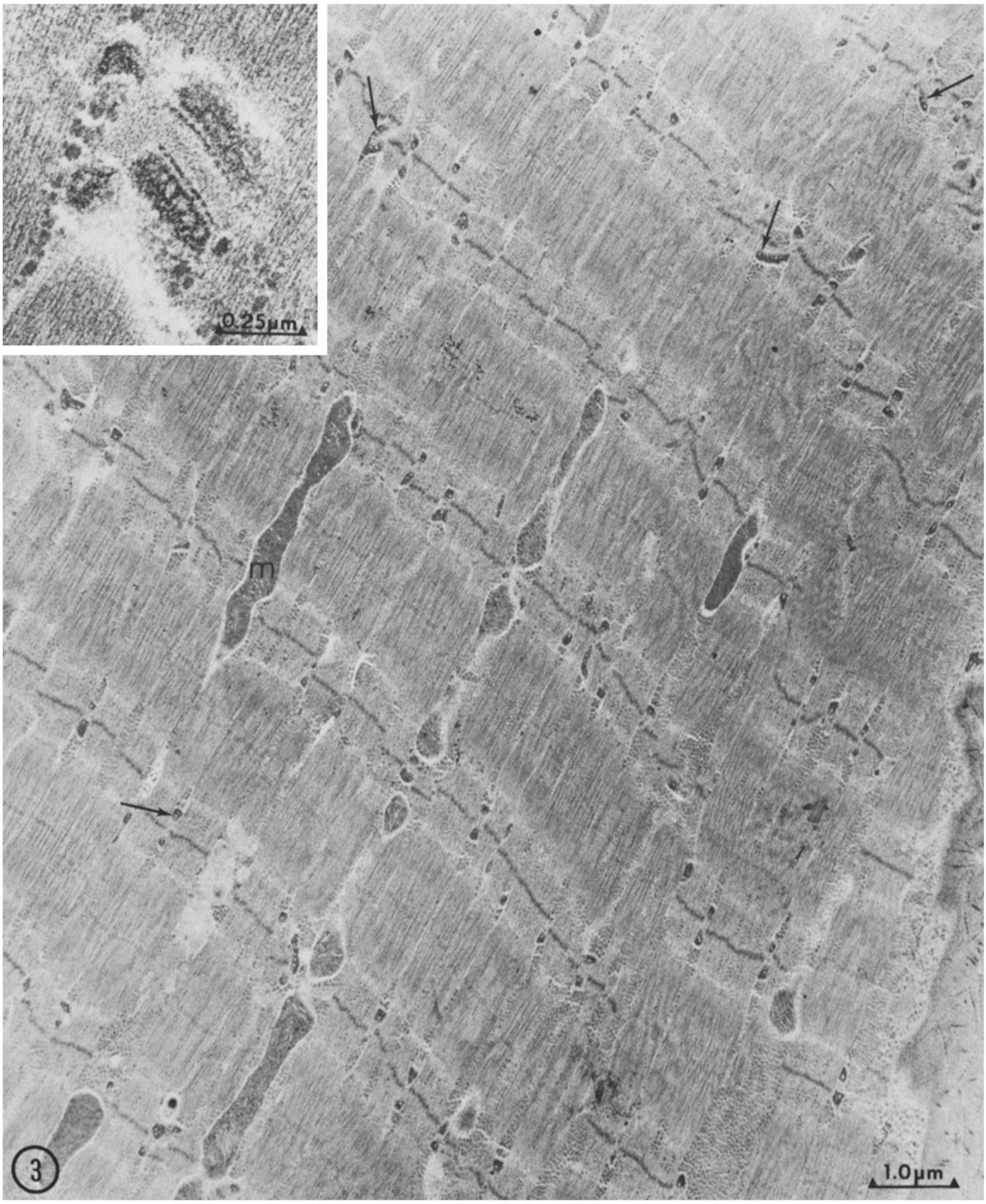


FIGURE 3 Longitudinal section of a resting muscle frozen and processed by freeze-substitution. Glycogen associated with the SR is seen between the fibrils. There are periodic electron-lucent circular regions in the TC along the junctional gap (see *inset*). × 14,400. *Inset*, × 60,000.

In previous studies (96) using small probes we have shown preservation of ionic gradients across membranes in cryosections. The presence of sharp gradients of K and Cl across the muscle cell membrane can also be demonstrated in x-ray maps with the present use of a high brightness field emission gun (Fig. 6).

By suitable astigmation of the electron beam, eliptically shaped regions of exclusively A-bands or I-bands, the latter

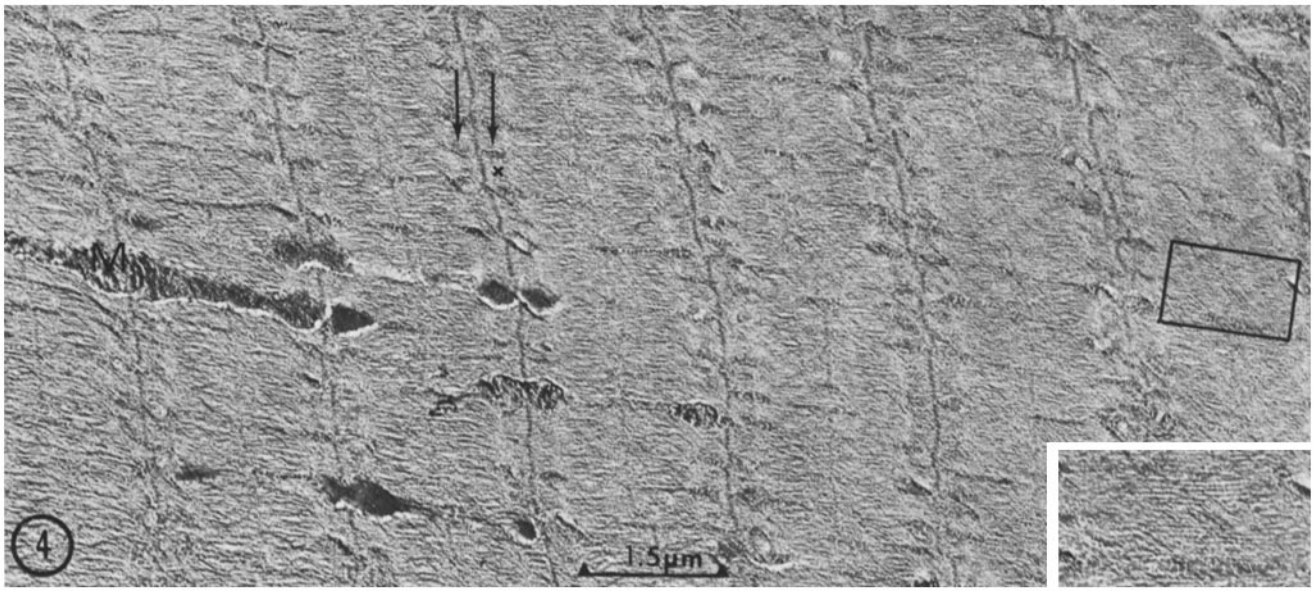


FIGURE 4 Longitudinal cryosection of a control muscle. Sarcomeres, Z- and M-lines, as well as SR, are apparent. Typical paired TC (arrows) such as those used for analysis and the adjacent cytoplasm in the I-band (*X*) are indicated. The right hand side of the image is toward the outer aspect of the bundle and has less ice-crystal damage than the left hand aspect. Note that myosin filaments can be imaged at the outer aspect (see *inset*). Mitochondria (*M*) and cristae can be seen. × 14,000.

including the Z-line, were analyzed (Fig. 5). The longest dimension of the probe was ~3–4 μm as illustrated in Fig. 5. In the resting muscles, a major portion of the 7 ± 2 SD mmol/kg dry wt whole fiber Ca was localized in the I-band. Included in the I-bands were Z-lines and several triads accounting for approximately a third of the sarcomere volume. After a 1.2 s tetanus, the total fiber Ca did not differ from the control values (Table I), but there was a shift in fiber Ca distribution (Fig. 5): the Ca concentration in the I-band decreased and in the A-band increased significantly ($P < 0.001$). These results, and earlier studies with radioautography (108, 109), obtained at relatively low spatial resolution, are consistent with the results of analysis with small probes (high resolution) described in the following section and showing that most of the fiber Ca is localized to the TC at rest and is redistributed during tetanus.

THE COMPOSITION OF THE TC AND OF THE ADJACENT I-BAND CYTOPLASM: The regions analyzed in a typical cryosection are illustrated in Fig. 4. The composition of 222 TC of 43 tetanized fibers and of 229 TC of 49 fibers from paired control muscles from the same 10 frogs is summarized in Table II with the results of individual experiments shown in Table III. The Ca concentrations of the control TC have a mean value of 117 mmol/kg dry wt. At 1.2 s after the onset of a tetanus, the Ca concentration of the TC was decreased by 59% (69 mmol/Kg dry TC wt). The Ca concentrations of both the resting and tetanized TC were normally distributed about the mean with the tetanized distribution being about half as wide as the resting one.

The K and Mg contents of the TC of the tetanized muscles were significantly increased ($P < 0.001$) when compared with their paired controls (Table II). The mean increases of 26 mEq Mg and 50 mEq K together are insufficient to compensate for the charge of the ~138 mEq Ca/kg dry TC released. The apparent charge deficit is 62 mEq/kg dry TC, with a 95% confidence limit of ±33 mEq/kg. The Na, Cl, and P concentrations of the resting and tetanized TC are not significantly different (Table II).

The ratio of the continuum counts over the TC and adjacent cytoplasm (a measure of relative dry mass) was 1.4 ± 0.5 SD ($n = 449$) in both tetanized and control fibers, indicating that there was no significant change in the dry mass of the TC during a 1.2 s tetanus. The water content of the TC, calculated (89) on the basis of 80% H_2O in the I-band and the continuum ratio (TC/cytoplasm) of 1.4, is 72%. This is significantly lower than the commonly quoted values (6 μl/mg) of H_2O in fragmented SR (21), but comparable to the water space (2.1 μl/mg protein) of heavy SR fractions containing a large proportion of TC (12). The sulfur content was slightly higher in the TC of tetanized muscles. Although statistically significant ($P < 0.02$), the physiological significance of this change, if any, is uncertain (see Discussion).

The results of paired analyses over the I-band adjacent to the TC using identical probe parameters (current density, probe size, and counting time) are summarized in Table IV with the individual experiments tabulated in Table V. The TC have higher ($P < 0.001$) concentrations of Ca, K, and P than the adjacent cytoplasm (Tables II and IV). The Mg concentration of the TC was significantly greater ($P < 0.001$) than the paired cytoplasmic contents, when comparing them on the basis of absolute number of atoms/volume, rather than concentrations. This is due to the fact that concentrations are proportional to the ratio of characteristic/continuum counts (see Materials and Methods) and the continuum counts are proportional to total dry mass. Given the 1.4 mass ratio of TC/cytoplasm, equally measured concentrations of a given element in the two regions will require a 40% larger number of atoms of that element in the TC. Conversely, higher measured concentrations of a cytoplasmic solute (e.g., Cl) may merely reflect a higher cytoplasmic water content. We can also estimate (based on 72% H_2O content of the TC and equilibration with a fiber K^+ of 129 mM)[1] that of the 554 mmol K/kg dry wt TC in control

[1] This estimate is the difference between the measured K content of the TC (554 mmol/kg dry wt) and the amount calculated to be dissolved in the 72% TC water on the basis of a concentration of 129 mM of K.

FIGURE 5 Longitudinal cryosection of a muscle frozen 1.2 s after the onset of a tetanus. The cell membrane and extracellular space can be seen at the lower edge of the figure. The dark longitudinal structures are mitochondria. Sarcomeres and Z-lines can be seen due to the differences in mass. The dashed lines indicate typical regions analyzed and the results of such analyses are shown below. Note that the astigmated spots over the I-bands cross several fibrils and thus include several TC. × 14,000.

$\bar{X}$ ± SD mmol/kg dry wt

	n	Na	Mg	P	S	Cl	K	Ca	continuum
Control									
I-band	10	43 ± 25	48 ± 18	306 ± 95	263 ± 82	55 ± 38	467 ± 139	24 ± 8	2,855
A-band	10	41 ± 19	51 ± 13	294 ± 68	303 ± 68	58 ± 38	438 ± 132	1± 2	2,713
Lg cyto	4	40 ± 24	46 ± 10	314 ± 65	290 ± 52	74 ± 41	466 ± 98	7 ± 2	
Tetanus									
I-band	12	45 ± 11	44 ± 8	294 ± 32	341 ± 16	73 ± 25	467 ± 48	10 ± 3	5,303
A-band	12	35 ± 13	42 ± 7	284 ± 39	368 ± 17	83 ± 31	455 ± 49	6 ± 3	4,981
Lg cyto	6	41 ± 23	37 ± 9	291 ± 32	343 ± 17	78 ± 18	458 ± 28	7 ± 1	

TABLE I

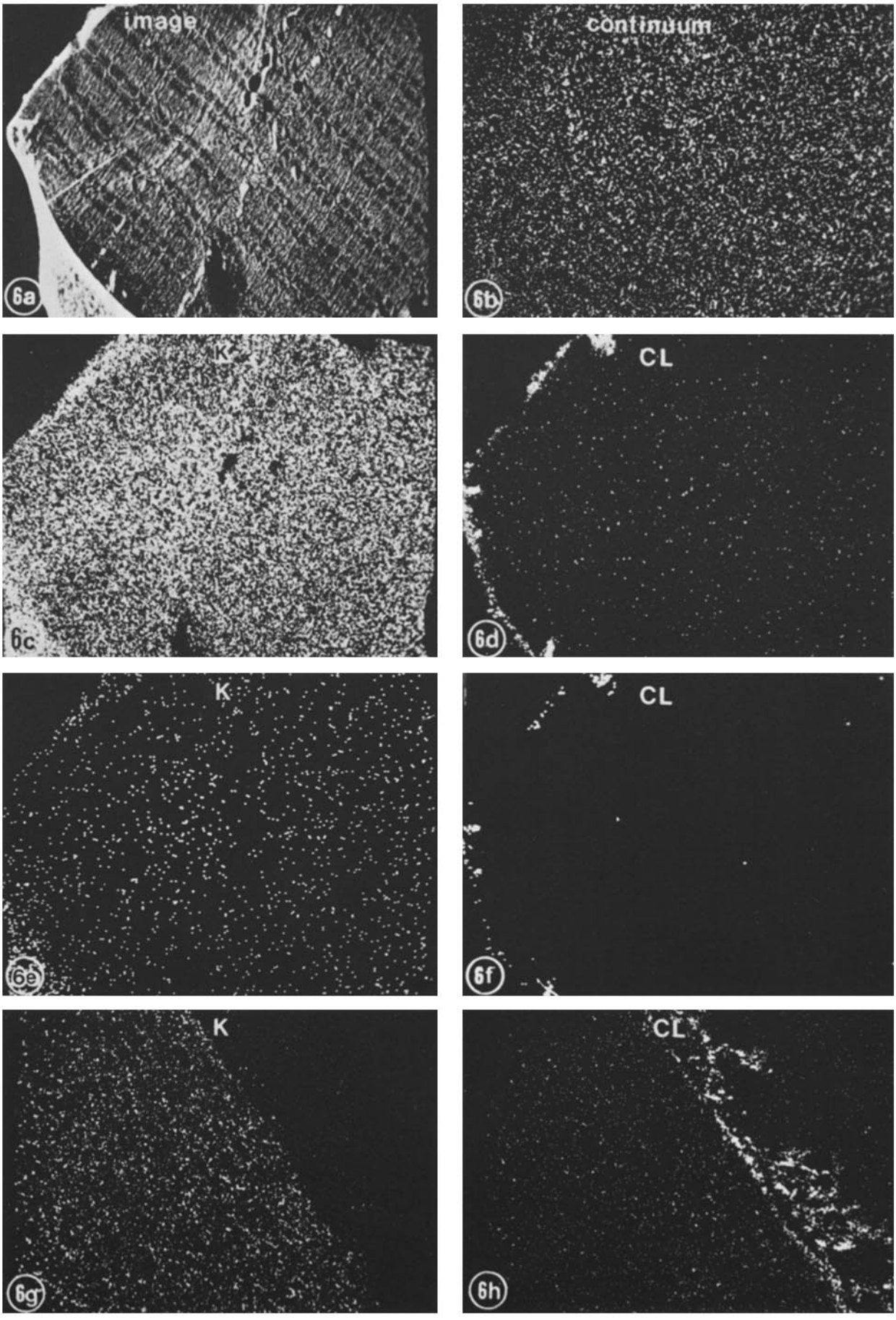
Elemental Composition of the Large Cytoplasm

	n	Na	Mg	P	S	Cl	K	Ca
Control	49	38 ± 23 (11)	41 ± 12	273 ± 60	236 ± 35	58 ± 26 (17)	431 ± 95 (129)	8.5 ± 4.6
Tetanus	43	33 ± 22 (10)	39 ± 12	267 ± 69	228 ± 52	58 ± 26 (17)	419 ± 92 (125)	8.6 ± 4.8

$\bar{X}$ ± SD mmol/kg dry weight. The concentrations expressed as mmol/l cell H_2O are shown in brackets and are based on a value of 77% cell H_2O.

muscles, ~222 were bound to anionic proteins. Control frog muscle 5 in Table III had a lower Ca and greater Mg concentration in the TC than the other nine control frog muscles. The cytoplasmic Ca concentration of this muscle also tended to be greater (Table V). In view of the findings on the tetanized muscles, it is possible that this muscle was slightly activated before freezing. It is unlikely that the fibers were damaged as Ca was not increased in the whole fiber analysis of this muscle.

The Ca content of the TC in resting muscles was higher than in our earlier study (96). Differences in frogs and the type of muscle used (frog toe muscle vs. semitendinous) may have contributed to this difference. We suspect, however, that the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use of phosphate Ringer in this study and in experiments on fatigued muscle (32) may have contributed to the higher Ca contents: a bicarbonate Ringer was used in our earlier experiments (96).

The cytoplasmic Ca concentration was 3.5 mmol/kg dry wt cytoplasm higher and the Mg concentration was lower in the tetanized muscles. The instrumental precision of the Mg measurements is lower than it is for elements having higher atomic numbers (87).

The cytoplasmic concentration of K measured with small diameter probes (Table IV) in the I-band (500–510 mmol/kg dry wt) was higher in both the resting and in the tetanized muscles than that measured with large diameter probes over several sarcomeres (Table I) (420–430 mmol/kg dry wt). Based on light interference microscopic measurements (45) of intact frog muscle fibers having an average of 77% cell H_2O content, it can be estimated that the H_2O in the I-band is 80%. Therefore, for an average internal K^+ of 129 mmol/l cell water, the calculated average concentration of K in the I-band is 516 mmol/kg dry wt, in rather good agreement with the experimentally obtained values.

The cytoplasmic Ca concentration in the I-band of the resting muscles was ~4.5 mmol/kg dry wt (Tables IV and V). Removal of the four rogue values (more than three SD greater than the mean) reduces this value to 4.2 mmol/kg dry wt, and subtracting the possible extra calcium due to contamination of the carbon films could reduce the value to as low as 3.8 mmol/kg dry wt. Analyses for long counting times (1,000 s) to reduce statistical errors were done on transverse sections of two resting muscles supported by carbon films ascertained to have no detectable Ca contamination; the Ca concentration measured in the I-band in these muscles was 3.1 mmol/kg ± 0.6 IEM (n = 5).

LONGITUDINAL SR: The composition of the LSR in tetanized muscles determined with focused, astigmated ~0.5-1 μm long × 25–30 nm wide, probes just sufficient to cover an LSR tubule, is shown in Table VI. The most significant finding is the lack of major sequestration of Ca in the LSR. The concentrations of Na, Mg, S, Cl, and K were not significantly different in, respectively, LSR and adjacent cytoplasm. The similarity of the K concentrations supports the notion that the "excess K" in the TC is bound to calsequestrin. The detection of small differences, if any, would require a considerably larger number of analyses, due to the statistical (Poisson) properties of the counting process. The results of several other analyses carried out during the course of this study, although not stored on the computer, were consistent with the data shown in Table VI in not showing high concentrations of Ca in the LSR.

The significantly higher P concentration of the LSR than the adjacent A-band is presumably due to the phospholipid content of the SR membrane. To test this premise, isolated heavy and light fractions of SR (66) were analyzed for P (Fig. 7). The light fraction contained 391 ± 28 SD mmol/kg dry wt in excellent agreement with the *in situ* analysis of 378 ± 67 SD and chemical P determinations of 390–420 mmol/kg dry wt (66). These findings are consistent with the major fraction of the microvolume of cryosection irradiated being composed of the LSR. Analysis of the isolated heavy fraction, including measurements with focused probes on individual TC, showed that the latter, but not the light fraction, contained high concentrations of Ca and Mg (Fig. 7).

MITOCHONDRIA: The composition of the mitochondria and most specifically their Ca content was not significantly different in tetanized and in control fibers. The concentrations of K, Na, and Cl were lower, on a dry weight basis, in the mitochondria than in the paired region of cytoplasm (Table VII). However, the greater number of x-ray continuum counts obtained (with identical probe parameters) in mitochondrial analyses compared to the adjacent cytoplasm indicates that the mitochondria are less hydrated. Assuming that the average cytoplasmic water is 77% (cytoplasmic paired analysis for the mitochondria included the A-band), the degree of hydration of the mitochondria, computed from the ratio of x-ray continuum counts obtained from the mitochondria and adjacent cytoplasm, is 65%. Assuming further that mitochondrial Na, K, and Cl are in solution, the concentration of ions in mitochondrial water can be estimated. The mitochondrial/cytoplasmic ratios (Tables IV vs. VII) of these concentrations in H_2O are 1.1, 0.9, and 0.5, respectively, indicating the absence of major mitochondrial/cytoplasmic gradients for these ions. The P concentrations were consistently higher in the mitochondria than in the adjacent cytoplasm. The mitochondrial/cytoplasmic continuum ratios of the control and tetanized muscles were not significantly different, indicating that there was no significant mitochondrial volume change during a 1.2 s tetanus. In one badly damaged fiber the mitochondria contained granules consisting of Ca and P.

DAMAGED FIBERS: Four damaged fibers were identified from the entire population of fibers examined. Damage was established on the basis of reversal of or significant reduction in the cytoplasmic Na and K gradients. One of these fibers is illustrated in Fig. 8 *a*. Dense deposits of Ca associated with P were localized to the TC only. Analysis of these deposits showed molar concentrations of Ca and P. For example, one of the deposits in Fig. 8 indicated by an arrow contained 1.4 mol Ca and 1.3 mol P/kg dry wt. Using a 6-μm diameter probe over this fiber, the measured Ca concentration (57 mmol/kg dry wt) was seven times greater than the average Ca concentration of undamaged fibers. Deposits were not found in the LSR in the three damaged fibers examined using focused probes. This was confirmed in Ca and P x-ray maps (Fig. 8 *b* and *c*) obtained with the high brightness field emission electron source, in which the individual TC of the triad can be resolved. The mitochondrion did not accumulate Ca and was therefore observed in the P, but not in the Ca x-ray map.

KCl CONTRACTURES: In three muscles frozen at 8,8 and 30 s after substitution of 80 mM KCl for NaCl in Ringer's solution, analysis of 45 TC from the contracted muscles and 32 from control muscles showed that 55% or 50 mM Ca/kg dry wt was released from the TC. The Ca release was accompanied

FIGURE 6 X-ray maps of resting frog semitendinous fibers, illustrating sharp gradients of K and Cl across the sarcolemma of rapidly frozen, cryosectioned, and freeze-dried muscle. The scanning transmission image was taken with one pass using 512 lines, 1 s/line and 512 picture elements/line. Note that the left hand edge of the fiber (*a*) with its rim of extracellular material has curled back on itself. X-ray maps of the same region taken with two passes are shown in *b*–*f*. The K and Cl maps shown in *c* and *d* include counts arising from the x-ray continuum which has been subtracted in *e* and *f*. Note the sharp demarcation of Cl in the rim of extracellular region (*d* and *f*) and that K is uniformly distributed between the center and edge of the fiber (*c* and *e*). X-ray maps of K and Cl from another fiber are illustrated in *g* and *h*. There is no evidence of K or Cl diffusion fronts at the edge of the fiber.

TABLE II

Elemental Composition of the Terminal Cisternae

	n	Na	Mg	P	S	Cl	K	Ca
Control	229	56 ± 39	59 ± 22	415 ± 82	214 ± 40	43 ± 19 (17)	554 ± 138	117 ± 48
Tetanus	222	58 ± 37	72 ± 23*	413 ± 82	225 ± 56	42 ± 20 (16)	604 ± 103*	48 ± 20*

$\bar{X}$ ± SD mmol/kg dry wt. Effect of tetanus (mEq): −138 Ca + 50 K + 26 Mg = −62 mEq*. The concentrations expressed as mmol/l cell H_2O are shown in brackets and are based on a TC H_2O content of 72% taken from a ratio of 1.4 for the TC: small cytoplasm continuum measurements and an 80% I-band H_2O content.
* $P < 0.001$

TABLE III

Composition of the Terminal Cisternae of Paired Control and Tetanized Frog Semitendinosus Muscles

Frog	Date	*n* TC	Na	Mg	P	S	Cl	K	Ca
					mmol/kg dry wt ± SD				
Control									
1	04-27-78	27	49 ± 32	60 ± 18	384 ± 66	239 ± 41	30 ± 11	504 ± 100	134 ± 52
2	05-01-78	29	39 ± 23	63 ± 17	401 ± 66	228 ± 33	52 ± 20	561 ± 86	121 ± 54
3	05-05-78	21	51 ± 29	52 ± 14	431 ± 66	215 ± 41	44 ± 14	595 ± 112	133 ± 61
4	05-08-78	28	64 ± 39	40 ± 17	425 ± 77	205 ± 29	35 ± 24	481 ± 78	120 ± 40
5	05-22-78	21	61 ± 35	91 ± 27	399 ± 41	217 ± 22	45 ± 11	621 ± 72	74 ± 20
6	07-11-78	40	65 ± 65	55 ± 18	534 ± 78	219 ± 46	53 ± 23	798 ± 122	111 ± 42
7	07-16-78	11	90 ± 43	56 ± 21	397 ± 58	220 ± 33	53 ± 20	570 ± 130	114 ± 31
8	08-16-79	15	41 ± 34	52 ± 13	435 ± 42	171 ± 27	51 ± 20	545 ± 61	115 ± 41
9	12-19-79	24	64 ± 28	59 ± 19	347 ± 77	180 ± 31	42 ± 17	436 ± 104	138 ± 48
10	01-23-80	13	54 ± 39	77 ± 22	405 ± 48	228 ± 36	32 ± 10	503 ± 73	112 ± 37
Grand weighted mean ± SD		229	56 ± 39	59 ± 22	415 ± 83	213 ± 41	43 ± 19	554 ± 138	117 ± 48
Tetanus									
1	04-27-78	19	59 ± 33	75 ± 17	361 ± 49	253 ± 31	28 ± 14	539 ± 75	37 ± 15
2	05-01-78	12	60 ± 33	68 ± 19	463 ± 49	296 ± 53	72 ± 22	678 ± 84	44 ± 19
3	05-05-78	24	56 ± 47	68 ± 17	418 ± 63	226 ± 28	52 ± 15	604 ± 85	39 ± 14
4	05-08-78	27	46 ± 33	63 ± 21	493 ± 72	244 ± 54	35 ± 13	618 ± 80	55 ± 27
5	05-22-78	27	63 ± 34	83 ± 25	434 ± 56	227 ± 35	48 ± 20	674 ± 99	51 ± 17
6	07-11-78	38	45 ± 29	66 ± 21	482 ± 49	215 ± 41	50 ± 21	658 ± 107	51 ± 25
7	07-16-78	9	50 ± 16	63 ± 15	435 ± 44	214 ± 18	34 ± 13	564 ± 56	48 ± 17
8	08-16-79	23	62 ± 39	84 ± 29	385 ± 57	132 ± 32	39 ± 13	565 ± 114	50 ± 19
9	12-19-79	17	63 ± 32	78 ± 18	360 ± 48	232 ± 23	27 ± 9	560 ± 75	56 ± 19
10	01-23-80	26	94 ± 42	71 ± 29	300 ± 56	291 ± 45	39 ± 19	544 ± 87	46 ± 17
Grand weighted mean ± SD		222	58 ± 37	72 ± 23	413 ± 82	225 ± 56	42 ± 20	604 ± 103	48 ± 20

by a gain ($P < 0.01$) in Mg (control 46 ± 27 SD, KCl contracture 61 ± 20 SD mmol/kg dry wt) in the TC.

The K content was higher in both the TC (748 ± 103 SD, $n = 45$) and in the paired cytoplasm (569 ± 180 SD, $n = 42$) than in either the resting or in the tetanized muscles. In view of the limited number of experiments in which, furthermore, the controls were not from the same frogs, as well as the fact that the fibers were presumably swelling (KCl was substituted for NaCl), we do not feel that calculation of charge balance is warranted in these experiments.

DISCUSSION

Calcium Release and Other Changes in the Composition of the TC during a Tetanus

The major finding of our study was that ~59% of the Ca content of the TC is released during a 1.2 s tetanus, and that this is accompanied by an increase in the concentration of other cations (K, Mg) in the TC. This increase is significant, but insufficient to account for the electrical charge represented by the amount of Ca released; the remaining apparent charge deficit is ~62 mEq/kg dry wt TC. Our demonstration of Ca release from the TC of activated muscle is in agreement with previous studies by a variety of other methods (108; for review, see reference 24). The total amount of Ca released during a tetanus (69 mmol Ca/kg dry TC), measured directly by electron-probe analysis, was unexpectedly high: it is sufficient to increase the total cytoplasmic Ca by ~0.9 mM. This exceeds by some two orders of magnitude the rise in free cytoplasmic Ca^{2+} measured in contracting muscle with Ca sensitive indicators (1, 8, 69), and is considerably more than the ~0.2 mmol Ca/kg wet fiber required to be bound to the Ca-specific sites on troponin (30, 105). The errors of our measurements are small (87, 89) relative to the amount released, and the amount released is also internally consistent with the measured increase in cytoplasmic Ca and with the concentration of Ca-binding proteins in the frog myoplasm (see below). The increase in the Mg content of the TC during tetanus is similarly consistent with the decrease in cytoplasmic Mg (Tables IV and V). However, not only were the uptakes of either K or Mg insufficient to account as counterions for maintaining charge neutrality during Ca release, but even the gain of these two cations together failed to maintain bulk electroneutrality. Given the

TABLE IV

Elemental Composition of the I-Band Cytoplasm (Analysis with Small Probes)

	n	Na	Mg*	P	S	Cl	K	Ca‡
Control	229	45 ± 44 (11)	54 ± 18	339 ± 80	263 ± 52	55 ± 27 (14)	510 ± 112 (128)	4.5 ± 7.1
Tetanus	222	40 ± 33 (10)	47 ± 18	338 ± 76	256 ± 65	54 ± 26 (14)	505 ± 108 (126)	8.0 ± 7.5

$\bar{X}$ ± SD mmol/kg dry weight. The concentrations expressed as mmol/l I-band H_2O are shown in parentheses and are based on a value of 80% H_2O in the I-band.

* $P < 0.001$, Δ −7.0 ± 3.3.

‡ $P < 0.001$; Δ +3.5 ± 1.3.

TABLE V

Composition of the I-Band Cytoplasm of Paired Control and Tetanized Frog Semitendinosus Muscles

Frog	Date	n	Na	Mg	P	S	Cl	K	Ca
					mmol/kg dry wt. ± SD				
Control									
1	04-27-78	26	35 ± 26	58 ± 19	330 ± 74	270 ± 51	40 ± 14	489 ± 101	1 ± 4
2	05-01-78	30	33 ± 30	57 ± 19	323 ± 69	272 ± 59	58 ± 17	513 ± 105	5 ± 6
3	05-05-78	21	49 ± 53	53 ± 16	328 ± 80	224 ± 29	67 ± 17	574 ± 106	7 ± 7
4	05-08-78	28	63 ± 46	45 ± 18	339 ± 81	242 ± 48	57 ± 48	471 ± 81	3 ± 8
5	05-22-78	21	42 ± 38	50 ± 15	313 ± 58	285 ± 40	54 ± 13	491 ± 69	10 ± 7
6	07-11-78	40	55 ± 64	58 ± 15	442 ± 71	273 ± 48	68 ± 32	644 ± 94	6 ± 9
7	07-16-78	11	78 ± 34	63 ± 16	352 ± 73	318 ± 37	69 ± 32	512 ± 130	3 ± 8
8	08-16-79	15	27 ± 29	56 ± 16	311 ± 41	253 ± 29	63 ± 20	484 ± 47	6 ± 6
9	12-19-79	24	37 ± 40	54 ± 19	308 ± 55	236 ± 49	52 ± 17	436 ± 101	2 ± 6
10	01-23-80	13	44 ± 32	43 ± 24	297 ± 73	307 ± 50	23 ± 11	449 ± 97	4 ± 6
Grand weighted mean ± SD		229	45 ± 44	54 ± 18	339 ± 80	263 ± 52	55 ± 27	510 ± 112	4.5 ± 7.1
Tetanus									
1	04-27-78	19	28 ± 32	41 ± 11	286 ± 48	263 ± 37	33 ± 8	448 ± 62	8 ± 9
2	05-01-78	12	47 ± 19	63 ± 10	412 ± 42	344 ± 51	94 ± 18	678 ± 78	9 ± 7
3	05-05-78	24	55 ± 33	44 ± 16	321 ± 61	224 ± 34	71 ± 19	513 ± 76	8 ± 9
4	05-08-78	27	29 ± 29	47 ± 13	406 ± 84	295 ± 44	53 ± 23	534 ± 95	6 ± 8
5	05-22-78	27	43 ± 37	54 ± 25	381 ± 68	296 ± 36	62 ± 24	592 ± 96	13 ± 9
6	07-11-78	38	41 ± 29	50 ± 13	371 ± 46	240 ± 50	66 ± 24	543 ± 89	6 ± 5
7	07-16-78	9	34 ± 17	47 ± 9	347 ± 58	242 ± 37	38 ± 7	513 ± 42	6 ± 6
8	08-16-79	23	26 ± 31	47 ± 15	271 ± 56	168 ± 35	41 ± 9	413 ± 86	11 ± 7
9	12-19-79	17	35 ± 40	40 ± 21	323 ± 46	264 ± 47	23 ± 8	436 ± 81	4 ± 4
10	01-23-80	26	54 ± 42	40 ± 25	274 ± 65	310 ± 78	50 ± 26	432 ± 93	7 ± 6
Grand weighted mean ± SD		222	40 ± 33	47 ± 18	338 ± 76	256 ± 65	54 ± 26	505 ± 108	8.0 ± 7

TABLE VI

Elemental Composition of the Longitudinal SR during Tetanus

	n	Na	Mg	P*	S	Cl	K	Ca	Continuum
LSR	11	42 ± 20	37 ± 14	378 ± 67	343 ± 57	46 ± 25	410 ± 113	7.7 ± 2.4	2537
A-cyto	11	34 ± 16	43 ± 11	280 ± 66	347 ± 60	67 ± 35	435 ± 95	5.1 ± 3.0	2230

mmol/kg dry weight ± SD.

* $P < 0.01$

apparent charge deficit of 62 mEq/kg dry wt during tetanus, a TC surface-to-volume ratio of 13 μm^{-1} (72) and an (assumed) TC membrane capacitance of 1 $\mu F/cm^2$, the movement of this amount of uncompensated charge would lead to a transSR potential difference of 13 V. For a 7-nm thick membrane, this would represent a potential gradient of ~19 million V/cm, clearly in excess of the dielectric breakdown value for biological materials (42). Indeed, the development of a −60 to −90 mV potential generated by the movement of 0.3–0.4 mEq/kg dry TC uncompensated charge across the SR would inhibit Ca release. Therefore, the most probable conclusion to be drawn from our results is that part of the charge neutralization during tetanic Ca release is due to the movement of counter and/or coions that are not measurable by energy dispersive electron-probe analysis. We have considered the possibility that inward movement of protons into the TC may be involved in this process (90), and it seems teleologically attractive to suggest that protons produced through ATP breakdown by the Ca-pump ATPase of the SR may be used for this purpose. Recent studies on fragmented SR have indicated that these membranes are permeable to protons (68) and that the latter can be ejected from the SR during Ca uptake (14). We also cannot exclude the possibility that organic anions are released from or cations move into the TC during Ca release, because elements in the

TABLE VII

Elemental Composition of the Mitochondria and Paired Small Cytoplasm

	n	Na	Mg	P	S	Cl	K	Ca
Mitochondria								
Control	39	23 ± 25 (12)	23 ± 13	394 ± 72	251 ± 35	12 ± 9 (6)	214 ± 60 (115)	1.7 ± 3.5
Tetanus	41	23 ± 19 (12)	22 ± 9	403 ± 72	259 ± 40	13 ± 8.4 (7)	218 ± 60 (117)	0.5 ± 3.7
Paired small cytoplasm								
Control	40	40 ± 33	53 ± 17	359 ± 96	268 ± 56	56 ± 20	526 ± 121	3.0 ± 5.8
Tetanus	41	41 ± 32	50 ± 17	348 ± 74	267 ± 56	53 ± 26	499 ± 126	7.2 ± 5.0*

$\bar{X}$ ± SD mmol/kg dry wt. The concentrations expressed as mmol/l mitochondrial H_2O are shown in brackets and are based on a mitochondrial H_2O content of 65%.

* $P < 0.001$

atomic table below Na are generally not detected with energy dispersive detectors having beryllium windows. The P content of the TC was not significantly different in the tetanized muscles, suggesting that phosphate was not a major coion moving with Ca, although extensive Ca-loading of the TC in damaged frog muscle fibers is associated with a parallel increase in P content (Fig. 8). Studies on isolated systems (111) suggest that when P_i is transported into the SR (41), it moves through a pathway other than the Ca-ATPase.

The increase in the Mg content of the TC during a tetanus may reflect an increase in the permeability of the SR membrane to this cation, given the reported low Mg permeability of isolated SR membranes (73).[2] Mg movement may take place through the open Ca channel or more specific Mg channels. However, because our measurements were made at the end of a 1.2 s tetanus, we cannot exclude the possibility that the increase in the Mg content of the TC may have reflected recovery processes (i.e., consequences of Ca pumping), rather than counterion movement during Ca release. It is anticipated that this question will be resolved in further studies of muscles frozen following brief activation at lower temperatures to inhibit the SR Ca-pump.

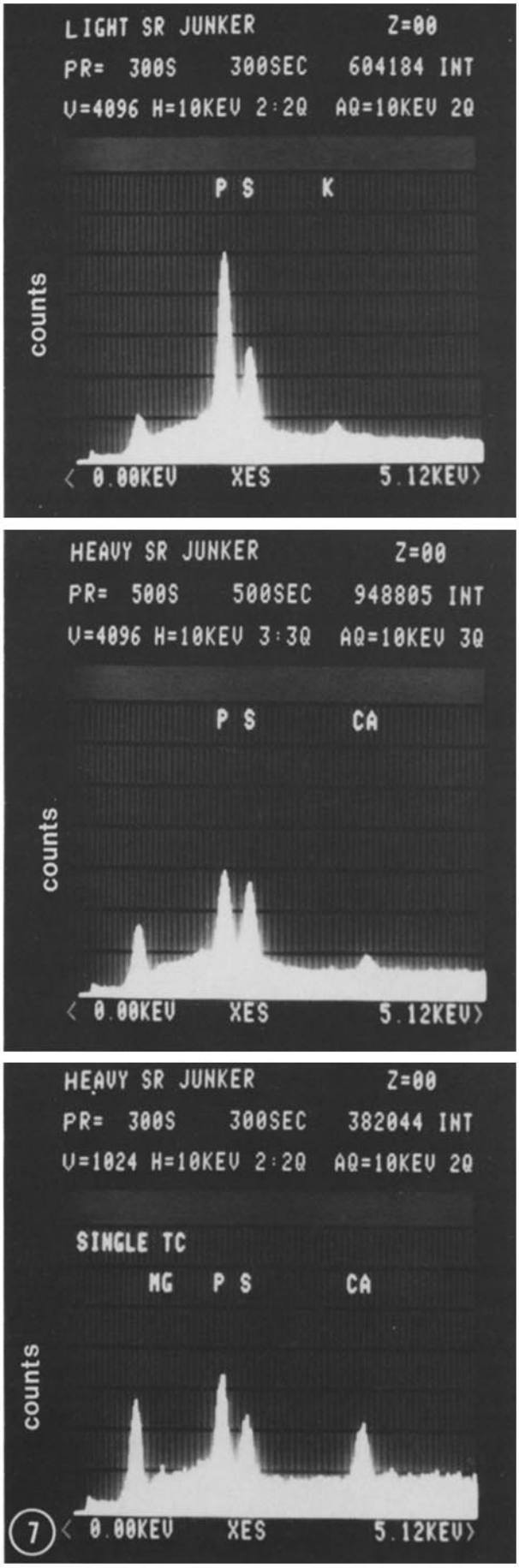
We observed a puzzling, but nevertheless statistically significant ($P < 0.02$) increase in the S content of the TC as a result of tetanus. Taurine is probably the main diffusible S compound in frog myoplasm and is negatively charged at physiological pH (49). Therefore, its inward movement would have to be against an (inside TC negative) electrical potential due to E_{Ca}. We have considered the possibility that the change in measured S content could be an artifact of radiation damage. However, vaporization of S through radiation damage (19) generally does not occur at cryogenic temperatures (89). Additionally, there is no known reason for a change in S content due to radiation damage to be more extensive in the tetanized than in control muscles. Quantitation of small changes in S content is difficult, unless special precautions are taken to eliminate the slight contamination of sections by S contained in the rotary pump oil. Contamination may not be detected in a single experiment. The results of individual experiments (Table III), however, show that the S content of the TC is higher in the tetanized than in the control pair in seven out of ten experiments. Therefore, we can not reject the possibility that the measured changes in S may be real and reflect underlying physiological processes.

[2] However, A. Scarpa (personal communication) finds that the space accessible to Mg is identical to the space available to water within the measurement time of 25 s.

Functional Implications: Excitation-Contraction Coupling and TransSR Potential

The mechanism through which the electrical potential change in the T-tubule triggers the release of Ca from the adjacent TC is not known (for review, see reference 24), leaving the door open for several theories of excitation-contraction coupling. These can be classified into three major mechanisms; (*a*) directly electrically coupled through ionic current flow into the SR (64); (*b*) charge movement coupled through dipole movement of fixed charges in the triadic junction (2, 86); and (*c*) diffusible transmitter (e.g., Ca^{++}) coupled (7, 26, 27). It is possible, of course, for charge movement to release a diffusible transmitter and any of the above mechanisms may be further coupled to Ca release through depolarization of the SR (11, 25, 71, 101). The first of these hypotheses envisions direct depolarization of the TC membrane by current flow from the T-tubules through the opening of nonspecific ionic channels that are closed at rest, but open during activation (22, 64). We found no significant change in either the Na or Cl content of the TC during activation, but the expected changes are below the limits of detectable difference. To be consistent with the measured passive electrical properties of striated muscle (16, 80), this hypothesis also requires that the TC be electrically insulated, perhaps at the intermediate cisternae (99, 100) from the longitudinal reticulum (64). The absence of Ca accumulation in the longitudinal tubules of the SR during tetanus fails to support this notion of ionic isolation. This evidence, however, is equivocal, because it may be argued that the Ca content of the LSR would not rise higher even if the latter were occluded at the level of the intermediate cisternae, due to the Ca pumping mechanism itself being inhibited at millimolar intraluminal free calcium concentrations (104). Thus, although our findings do not support a mechanism of excitation-contraction coupling based on the flow of ionic currents through the triadic junction, they cannot rule it out completely.

The absence of stoichiometrically compensating K movement into the TC as a counterion for Ca was somewhat surprising, in view of the relatively high permeability of fractionated SR to K (20, 53, 65, 67) and the apparently completely free distribution of this cation across the SR membrane in resting frog muscle (96, 97, and our study). An explanation for this may be found in observations that show voltage-dependent gating of K channels in the isolated SR (58, 70). Specifically, at pH 7 the K conductance of the SR channels incorporated into planar phospholipid bilayers is near zero at −50 and maximum at +100 mV applied potential. If this also occurred in the living fiber, it could result in the closing of the K



channels and reduced inward K current as the transSR potential tends to the Ca equilibrium potential (~−56 mV SR negative). Unfortunately, it is not known whether or how the cis-trans polarity of vesicles incorporated into phospholipid bilayers reflects the polarity of the *in situ* TC. We consider it unlikely that K accumulation into TC during tetanus may have been obscured by volume changes due to water movements or lost through freeze-drying for the following reasons: (*a*) there was no significant swelling or shrinkage of the TC profiles in freeze-substituted material and the TC/cytoplasmic dry mass ratio (as reflected in continuum counts) was unchanged during tetanus; (*b*) sharp in vivo ionic gradients across cell membranes are preserved during rapid freezing, cryoultramicrotomy and drying (35, 96, and our study), even in intracellular vacuoles (32, 96); (*c*) the gain in Mg content of the TC during tetanus was readily demonstrable, although it should be negligible compared to K uptake if the respective permeabilities, as measured in isolated preparations, dominated the influx mechanisms; and (*d*) the gain in K in the TC during K contracture was preserved by the preparative cryotechniques (our study) as were comparable changes in mitochondria and in nuclei in vascular smooth muscle (89).

The magnitude and sign of the electrical potential across the SR membrane has considerable bearing on the mechanism of Ca release. Although electron-probe analysis measures total elemental content, rather than the ionic activities relevant for determining diffusion potentials, we can place some upper bounds on the potentials that could be generated by taking advantage of the known affinities of the cation-binding proteins in the TC (46, 78). In resting muscle, the excess of K in the TC over that found in the cytoplasm is probably due to binding to calsequestrin, while the Na and Cl contents of these two compartments are not significantly different. Thus, none of these elements would give rise to a large (>5 mV) transSR electrical potential. Contributions from Ca and Mg would also be negligible in view of the very low permeability of the resting SR to divalent cations (73, 102). Therefore, we conclude that, barring the contribution of organic ions, the transSR potential in resting muscle is very small (≤5 mV) or nonexistent.

In activated muscle, birefringence and Nile blue fluorescence signals have been interpreted as showing a change in the transSR potential (3, 6, 56, 76, 103). The change in fluorescence persists throughout tetanic stimulation, albeit at a level reduced from its peak value (6). If such a potential change occurred, its most likely source would be the increase in the Ca permeability of the SR that results in Ca release. It can be estimated that, from an initial transient, E_{Ca} would tend to a value of ~−56 mV with the SR inside negative (assuming maintained free Ca concentrations of 10^{-3} and 10^{-5} M in, respectively, SR and cytoplasm). We note that, assuming that the selectivity of the cation-binding sites of calsequestrin is unchanged, the increased binding of K and Mg to these sites implies some fall in the free Ca^{++} and possibly a rise in free Mg^{++} in the SR during tetanus. The affinity of isolated calsequestrin for Ca^{++} ($K_d \cong$

FIGURE 7 X-ray spectra of the light SR (upper), heavy SR (middle), and of an individual TC in the heavy SR fraction (lower). The number of counts on the ordinate are shown for the energies in KeV on the abscissa. The elements giving rise to the characteristic peaks are indicated. The spectra of the heavy fraction and of the single TC showed them to contain high concentrations of Ca. The spectrum of the single TC also includes a small but significant Mg peak.

800 μM in the presence of 0.1 M KCl (46, 47, 78) is about five times higher than for Mg and some two orders of magnitude higher than for K (46).

We may now consider the consequences of a change in the SR potential from 0 to −60 mV (E_{Ca}) on the distribution of diffusible ions and, in this context, the implications of our results. The equilibrium redistribution of fiber K according to a transSR potential of −60 mV would drive the intraSR concentration of K^+ to ~730 mM at the expense of the cytoplasmic K concentration which would fall to ~73 mM.[3] Experimentally, we find K uptake into the TC to be much smaller than this value, in fact, less than the amount that would be required to replace the released Ca on the calcium-binding proteins. Furthermore, there is no electrophysiological evidence of such large changes in cytoplasmic K^+ during tetanus.

The unchanged Cl content of the SR also argues against a large maintained SR potential during tetanus, because this permeant ion (15, 20, 53, 67) would be expected to distribute electrophoretically according to the developed potential. A transSR potential of −56 mV due to E_{Ca} would result in a tenfold chloride concentration gradient across the SR membrane. Given a Cl permeability of $\sim 5 \times 10^{-6}$ cm/s (15), we would expect a steady-state distribution to be reached during the 1.2 s tetanus. Barring the unlikely possibility of extensive chloride binding in the TC or a much lower chloride (and K) permeability of the *in situ* than of the isolated SR, our results suggest that tetanic Ca release is not associated with large or sustained changes in transSR potential. Nonelectrogenic movements of Ca are also thought to occur in fragmented SR preparations (4). A reduction in the K permeability of the SR during tetanus could be caused by the developed SR potential itself or by changes in pH or in cytoplasmic free Ca concentration (58, 70). In addition to the movement of protons or organic ions, the potential due to E_{Ca} may also be partially balanced by an electrogenic Ca pump (111) or electrogenic Mg^{++} flux into the SR.

The Composition of the Cytoplasm and of the LSR during Tetanus: Changes in Ca and Mg Content and Their Relation to Parvalbumin

The release of 69 mmol/kg dry TC wt Ca from a compartment representing 4–5% of the volume of the whole fiber (72, 79) would lead to an ~0.9–1.2 mmol increase in the total Ca in fiber water or a change of 3–4 mmol/kg dry wt fiber. The Ca content measured in the I-band was ~3.5 mmol/kg dry wt higher (Table IV) in the tetanized than in the resting muscles, in reasonably good agreement with the change in the Ca content of the TC. As noted earlier, the total amount of Ca released into the fiber is significantly greater than either the free Ca measured by indicators or the amount (~0.2 mmol/l fiber water) that is considered to be necessary to be bound to the regulatory sites of troponin (106). Frog muscle, however, contains ~0.35 mM parvalbumin (33), a low molecular weight soluble protein that has two Ca-binding sites with affinities considered to be identical to those of the two Ca/Mg sites of troponin (31, 33, 37, 83). Therefore, the total amount of Ca released from the TC is that expected to be bound to the Ca-specific sites on troponin (0.2 mmol/l) and on the combined Ca^{++}/Mg^{++} sites of troponin[4] (0.2 mmol/l) and parvalbumin (0.7 mmol/l). In this regard, it is interesting to note that the very high SR volume and Ca content of the ultrafast toadfish swimbladder muscle is associated with unusually high (~1.5 mM) parvalbumin content (40). Studies of isolated parvalbumin suggest that the Ca^{++}/Mg^{++} sites, as their name implies, can bind either of the two divalent cations, but that the exchange of one divalent cation for another is rate-limited by the relatively slow off-rates from parvalbumin (17, 37, 84). Thus, with the Mg bound to the Ca^{++}/Mg^{++} sites in resting muscle and with the halftime of its removal of ~550 ms (84), an exchange of Ca for Mg would not occur during a twitch, but only during a tetanus. In this case, the significant decrease in the cytoplasmic Mg content of tetanized muscle, and the reciprocal increase in the Mg content of the TC (Table II and III), may reflect the removal of Mg from parvalbumin and troponin, rather than a 30% decrease in the free Mg^{++} content (0.6 mM) (36) of frog muscle. These relatively large, and perhaps unanticipated changes in bound divalent cations observed during a tetanus may contribute to the "extra heat" of activated muscle (18, 43). The enthalpy of these reactions has been recently investigated (110).

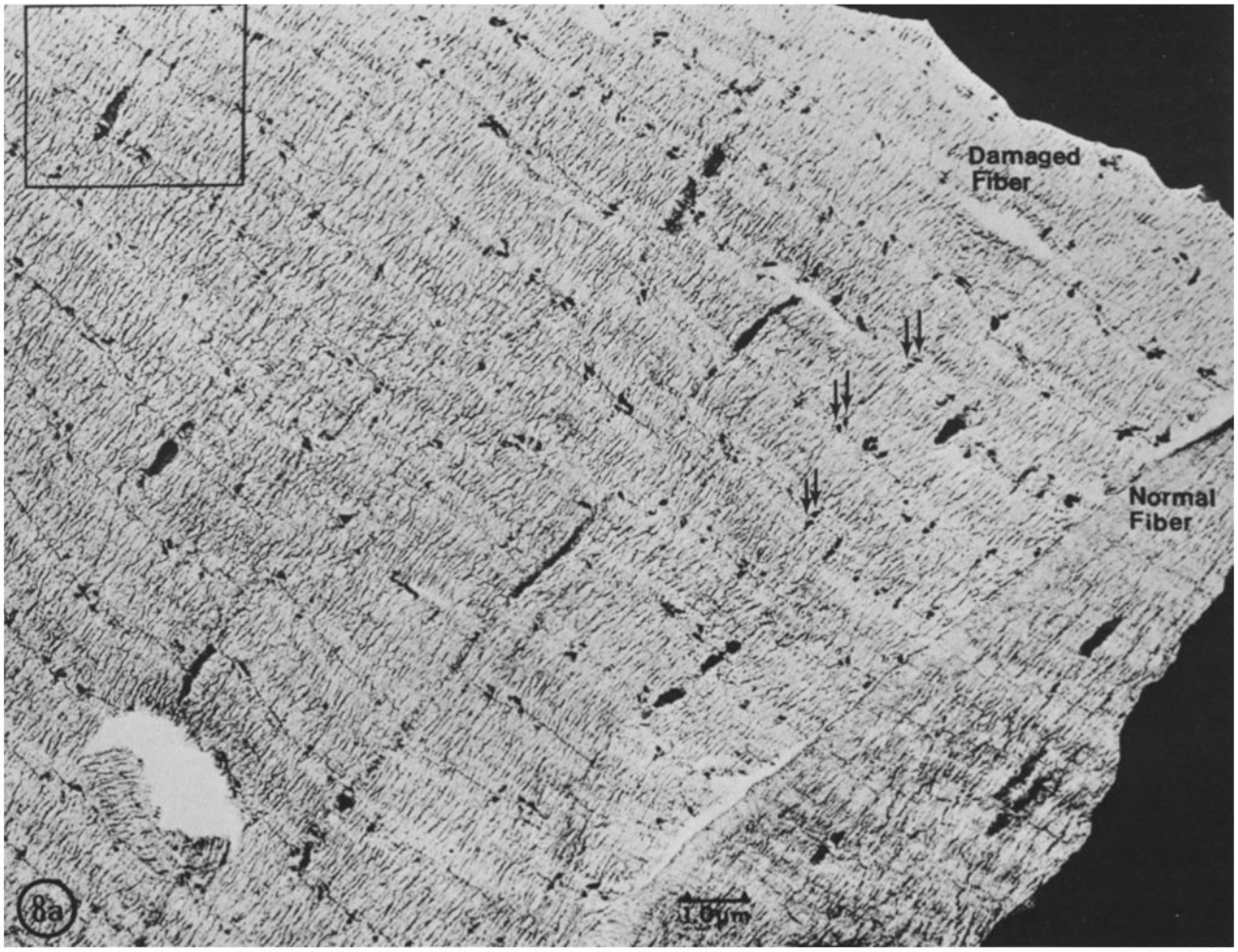
The Ca content of the tubules of the LSR was not significantly different from the adjacent cytoplasmic concentration. While small differences in Ca content, due to Ca binding by the Ca-ATPase or to a millimolar concentration dissolved in the luminal fluid, would not have been detectable by the statistical precision of these analyses, we can definitely exclude the possibility that the Ca concentration in the LSR is comparable to that in the TC. Thus, if the Ca released from the TC, excluding that bound to the regulatory sites on troponin (~0.2 mM), were sequestered in the LSR, the concentration in the latter would be ~44 mmol/kg dry wt LSR. The much lower observed values agree with the results (Table IV) of the analysis of the cytoplasm, showing that most of the Ca released during tetanus was distributed outside membraneous organelles. Even in fibers in which the TC were loaded with molar Ca and P (Fig. 8), the LSR was not loaded. Therefore, our results do not support the notion, based on earlier radioautographic studies having lower spatial resolution (108, 109), that Ca, after leaving the release sites (TC), is accumulated and stored for a relatively long time at "uptake sites" of the LSR. Our results suggest that ^{45}Ca, identified in radioautographs as bound to LSR (108, 109), was in fact bound to Ca-binding proteins. The combined results of the analysis of the TC and of the LSR are also inconsistent with the interpretation of Ca^{++} flux data that ascribe a relatively small (0.2 mmol/kg wet muscle), fast exchanging (time constant = 2.7 min) component as representing the Ca content of the TC, and a larger (0.54 mmole/kg wet muscle) slowly exchanging (time-constant = 1,244 min) component to the LSR (54). The relatively low Ca content of the LSR that, in contrast to the TC, contains little or no calsequestrin (12, 51, 52, 66), does support the conclusion that the free Ca^{++} in the SR is in the low millimolar range (104).

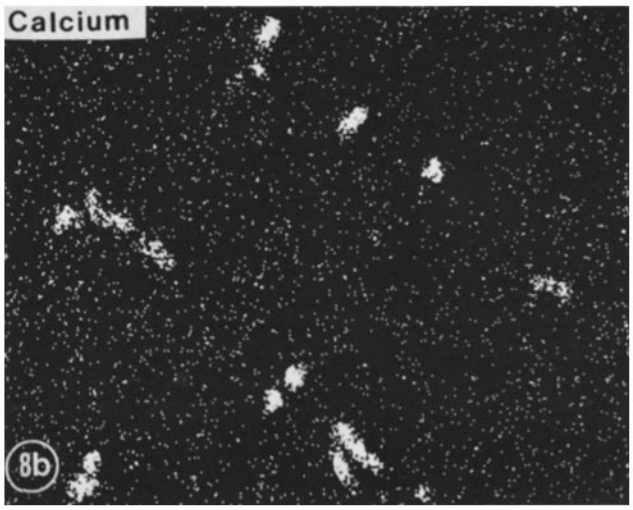
The Composition of Cytoplasm in Resting Muscle

The composition of the fibers measured with large diameter probes covering several sarcomeres, fibrils, and organelles re-

[3] To reach equilibrium in 1.2 s, however, would require a K^+ flux across the SR that is some three orders of magnitude higher than K^+ flux across the resting frog surface membrane. Such flux rates can be observed, albeit generally for only ms intervals during muscle or nerve action potentials (42).

[4] We note that the binding properties of, respectively, isolated and *in situ* troponin may be different and that one or both of the Ca/Mg sites of troponin maybe occupied by Ca in resting muscle.





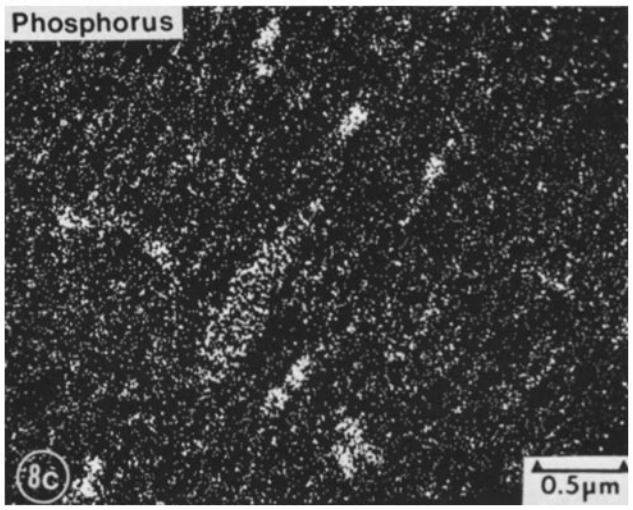


FIGURE 8 *a*, *b*, and *c*, longitudinal cryosection of a damaged frog semiendinosus muscle (containing abnormally high cytoplasmic [Na] and low [K]) in which the TC (arrows) of the SR are massively loaded with Ca and P as shown in the Ca and P x-ray maps in *b* and *c*, taken from the boxed area of *a*. Note the absence of deposits in the LSR. A mitochondrion is apparent in the P, but not in the Ca map. The individual TC of the triad are resolved in the Ca and P maps. The x-rays maps have not been corrected for the back ground counts which arise from the x-ray continuum counts and, in the Ca map, also from the K K_β signal.

mained unchanged during a 1.2 s tetanus (Table I). As previously indicated (96), the fiber K, Ca, P, Mg, and Na concentrations measured with electron-probe analysis are in very good agreement with independent bulk measurements on frog muscle.

The chloride content of these 15–20 fiber bundles was invariably higher than expected on the basis of a electrochemical equilibrium, possibly due to operation of a Cl pump, as has already been noted in our previous studies of resting muscle (96). Studies with ion selective microelectrodes suggest that cell

Cl increases in striated muscle after isolation (61), and as previously noted by Boyle and Conway (9), fibers gain Cl during storage.

The concentration of chloride measured with small probes in the I-band cytoplasm (Table IV) was not higher than the values obtained with large probes covering several sarcomeres (Table I). This differs from the distribution of K that was higher in the I-band, in proportion to the greater water content in this region (see Results, and 45). The observed distribution of chloride implies that it is present at somewhat higher concentrations in regions less hydrated in vivo than the I-band and included in the large area analyses. Because the mitochondria, if anything, exclude chloride (Table VII), our observations suggest that the chloride content is higher in the A-band than in the I-band. This conclusion, while still to be strengthened by paired analyses of the I- and A-band, is consistent with the substantial binding of chloride by isolated myosin (85) and with Donnan potential measurements of the A-band indicating the presence of associated chloride (23).

The cytoplasmic Ca concentration in resting muscle was unexpectedly high, considering the fact that magnesium, rather than Ca, is the bound divalent cation on actin in frog (55) as in mammalian muscle (107). Although the experiments were not expressly designed to detect very low concentrations of Ca, considerable precautions were taken in the analysis of the data to eliminate the possibility of this high value being due to artifact. The fact that mitochondrial concentrations measured simultaneously were lower than cytoplasmic (Table VI) and that electron-probe analysis of sections of Ca-free Spurr's resin showed them to have Ca concentrations not significantly different from zero value (T. Kitazawa. Unpublished observations.) suggest that the unexpectedly high cytoplasmic (obviously bound) Ca measured is not due to a systematic error. It obviously will require a larger number of experiments specifically designed to measure low concentrations of Ca (with long counts and relatively high currents to obtain better count statistics from a single analysis etc.) to unequivocally clarify this problem and answer such questions as which divalent cation occupies the Ca/Mg sites of troponin in resting muscle. The measured cytoplasmic Ca concentration, in the I-band, is consistent with one of the troponin and parvalbumin sites being occupied by Ca.

Mitochondrial Composition

Mitochondrial composition remained unchanged during the tetanus. The Ca content of mitochondria occupying 1.6% of fiber volume (72) accounts for only ~4 μmol Ca/kg wet fiber. This is much less than the 90 μmol/kg wet fiber tentatively allocated to mitochondria on the basis of ^{45}Ca flux studies (54). Mitochondrial Ca content was lower in tetanized than in resting muscle, although the difference was not statistically significant. Therefore, frog-muscle mitochondria, like those of other cells (89), do not sequester significant amounts of Ca during a physiological rise in cytoplasmic free Ca to levels that can maximally activate the contractile proteins. Similarly, mitochondrial Ca is not increased in severely fatigued frog skeletal muscle (32). The large "safety factor" between Ca accumulation by the SR and by mitochondria is also indicated by our observation of heavy Ca loading of the TC in the absence of significant mitochondrial Ca loading (Fig. 8). High mitochondrial Ca content in the form of granules occurs as a result and, in our opinion, as *prima facie* evidence of abnormally high cytoplasmic free Ca levels found in very damaged cells (34, 50, 89, 98, and this study). In this regard only cryosections can be used satisfactorily to identify damaged cells through their cytoplasmic monovalent ion composition, because both liquid fixatives and freeze-substitution remove the diffusible ions from the cytoplasm (13, 77, 93).

The monovalent ion content of the mitochondria was lower than the cytoplasmic content expressed on a dry weight basis in both the control and tetanized muscles, but this difference is presumably due to the lower degree of hydration of mitochondria. The calculated (from the continuum counts) water content of frog-muscle mitochondria was ~65% and remained unchanged during a tetanus. This value was similar to the value estimated with electron-probe analysis of *in situ* mitochondria in smooth muscle or obtained with direct measurements on isolated mitochondria (89). When Na, K, and Cl expressed as their concentrations in mitochondrial water are compared to the similarly computed cytoplasmic concentrations, the respective values are not significantly different. These results are again comparable to those obtained with mammalian vascular smooth muscle, and as, pointed out elsewhere (89), show that these ions are not distributed according to a large transmitochondrial potential.

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