ELECTRON PROBE ANALYSIS OF VASCULAR SMOOTH MUSCLE

Composition of Mitochondria, Nuclei, and Cytoplasm

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ABSTRACT

Electron probe analysis of dry cryosections was used to determine the composition of the cytoplasm and organelles of rabbit portal-anterior mesenteric vein (PAMV) smooth muscle. All analytical values given are in mmol/kg wt ± SEM.

Cytoplasmic concentrations in normal, resting muscles were: K, 611 ± 1.7; Na, 167 ± 2.7; Cl, 278 ± 1.0; Mg, 36 ± 1.1; Ca, 1.9 ± 0.5; and P, 247 ± 1.1. Hence, the sum of intracellular Na + K exceeded cytoplasmic Cl by 500 mmol/kg dry wt, while the calculated total, nondiffusible solute was ~50 mmol/kg. Cytoplasmic K and Cl were increased in smooth muscles incubated in solutions containing an excess (80 mM) of KCl.

Nuclear and cytoplasmic Na and Ca concentrations were not significantly different. The mitochondrial Ca content in normal fibers was low, 0.8 ± 0.5, and there was no evidence of mitochondrial Ca sequestration in muscles frozen after a K contracture lasting 30 min. Transmitochondrial gradients of K, Na, and Cl were small (0.9–1.2). In damaged fibers, massive mitochondrial Ca accumulation of up to 2 mol/kg dry wt in granule form and associated with P could be demonstrated.

Our findings suggest (*a*) that the nonDonnan distribution of Cl in smooth muscle is not caused by sequestration in organelles, and that considerations of osmotic equilibrium and electroneutrality suggest the existence of unidentified nondiffusible anions in smooth muscle, (*b*) that nuclei do not contain concentrations of Na or Ca in excess of cytoplasmic levels, (*c*) that mitochondria in PAMV smooth muscle do not play a major role in regulating cytoplasmic Ca during physiological levels of contraction but can be massively Ca loaded in damaged cells, and (*d*) that the *in situ* transmitochondrial gradients of K, Na, and Cl do not show these ions to be distributed according to a large electromotive Donnan force.

KEY WORDS electron probe analysis · smooth muscle · nucleus · mitochondrion · calcium · sarcoplasmic reticulum

Blood vessels represent a classical example of a multicellular preparation in which estimates of cellular ionic concentrations by flux techniques

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TABLE I

Experimental Solutions

Solution	Na	Mg	Cl	K	Ca	H_2PO_4	SO_4	HCO_3	Dextrose
					mM				
Krebs	143.8	1.2	132	5.9	1.2	1.2	1.2	18.7	5.6
Krebs + 80 mM K	143.8	1.2	212	85.9	1.2	1.2	1.2	18.7	5.6
K_2SO_4 No. 1	−*	1.2	17.4	179.2	2.5	1.2	76	16.0	5.6
K_2SO_4 No. 2	−*	1.2	23.2	158.2	2.5	1.2	76	−	5.6

All muscles had 4% bovine serum albumin (Sigma, No. 6,003, essentially fatty acid free) added to the final incubation solution (pH 7.4) for at least 30 min before freezing.

* Analysis of the K_2SO_4 solution after incubation of the muscles showed a residual [Na] of ~10 mM originating from bovine serum albumin and the specimen.

are often ambiguous because of the complex geometry, large extracellular spaces, and extensive extracellular binding sites of the tissue (for review, see references 29 and 52). In the present study, we used electron probe analysis of cryosections to determine the intracellular distribution and concentrations of monovalent ions[1] as well as the role, if any, of mitochondria in the regulation of cytoplasmic Ca in vascular smooth muscle[2] (64), because it is possible with this method to directly quantitate the composition of cells and cellular organelles *in situ* (e.g., 8, 19–22, 34, 35, 49, 50, 59, and 60).

We specifically wished to determine whether the excess Cl in smooth muscle (6, 30, 31, 33) is compartmentalized in organelles or distributed in the cytoplasm, and we compared nuclear and cytoplasmic composition to assess whether cell nuclei are sites of Na sequestration (for review, see references 7 and 40). We also obtained estimates of the transmitochondrial K, Na, and Cl gradients *in situ*, as these quantities may reflect the transmitochondrial membrane potential implied by the chemiosmotic hypothesis (39). In addition to providing answers to these questions, the analysis of our results suggests the existence of some unidentified polyvalent anion(s) in smooth muscle. Preliminary reports of some of our findings have been presented (51, 56–58).

MATERIALS AND METHODS

The portal-anterior mesenteric veins (PAMV) of 4- to 6-lb male rabbits were used. This vascular smooth muscle preparation has been well characterized in ion flux (30, 31), electrophysiological (53, 63), and ultrastructural (13) studies. Longitudinal strips were incubated at 37°C in a modified oxygenated Krebs' solution for 30–60 min, and then transferred to the experimental (contracture) solution. In several experiments, two vascular strips were prepared from the same vein. In this case, one strip was a paired control of the contracted strip and was incubated in the modified Krebs' solution throughout the experimental period. The composition of the solutions used is shown in Table I. Contracted muscles were maintained for 30 min in the high K contracture solutions, during which time a tonic component that was ~80% of the peak contraction was maintained (31). The solution with added KCl is hyperosmotic but (because of its Na content) designed to maintain the same volume as the muscles incubated in Krebs' solution (2, 31). This solution was chosen to avoid the swelling associated with the substitution of K for Na, while retaining Na as a physiological indicator of cell membrane permeability.

Strips were mounted in a specially designed stainless steel holder and frozen in supercooled Freon 22 at −164 ± 2°C in an apparatus previously described in detail (59). The freezing rates attained by this method (9) are faster than those obtained with other coolants, including liquid N_2 slush, and nearly as fast as the freezing rates attainable with liquid propane, but without the explosive hazard of the latter. Sections ~1,000- to 2,000-Å thick were cut on an LKB cryo-ultramicrotome (LKB Producter, Bromma, Sweden), modified to maintain an ambient temperature in the cryochamber of −130°C, at a specimen temperature of −110°C and knife temperature of −100°C. Removal of the sections from the knife, transfer to the Denton 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 study of striated muscle (59). All the tabulated electron probe data were obtained on unstained specimens; however, a few grids

[1] Although the electron probe measurement is insensitive to chemical and valence states, K, Na, and Cl will be referred to as ions in keeping with accepted terminology based on other methods.

[2] Although we use the generic term "vascular smooth muscle" for purposes of this Discussion, in view of the heterogeneity of smooth muscles, different smooth muscles may, and probably do, have somewhat different cellular composition.

were stained with osmium vapor in vacuo (for 10 min) to enhance contrast for illustrations.

Electron probe analysis was done on a Philips EM 301 transmission electron microscope having a 30-mm² Kevex Si(Li) x-ray detector (Kevex Corp., Foster City, Calif.) and computerized recording apparatus as described previously (59), or on a Philips EM 400 high vacuum electron microscope used in conjunction with the same detector and multichannel analyzer. All analyses were done with a liquid N_2-cooled holder, at −100° to −110°C in the EM 301 and at ~−165°C in the EM 400, to minimize contamination and radiation damage (24, 49; for review, see references 18, 27, and 49). In addition to specimens mounted on Cu grids, some specimens were also examined on Ti grids to eliminate possible interference caused by the overlap of the Cu L-line with the Na K-line. As expected, there was no significant difference between the results obtained with the two types of grid material, because the multiple least squares fitting routine readily resolves peaks separated by 100 eV in the energy region of 1,000 eV where the resolution of our detector is 130 eV (49). Therefore, the results obtained on the two types of grids are reported together.

The method used to convert x-ray spectra to concentrations is based on the fact the ratio of the characteristic peak counts to the counts in the x-ray continuum generated by the specimen is proportional to elemental concentrations (23); this has been described and validated in detail (49, 50). The statistical analyses were also described in a previous publication (49). Continuum counts (proportional to the total mass of the microvolume irradiated by the probe) are measured in the energy region of 1.34–1.64 keV. Because the continuum counts are directly proportional to specimen mass (related to section thickness) and to the probe currents used, direct comparisons of these counts should only be made where paired analyses were done using the same probe parameters within two adjacent regions of the same section (e.g., values presented as paired within Tables III–V). Continuum counts obtained on different specimens (e.g., cytoplasm of normal and contracted muscles in Table IV) should not be compared, because they are influenced by differences in section thickness and variations in probe current. Negative concentration values are the result of statistical fluctuations and have no biological significance (59). The SE is given as SD = $\sqrt{n\,\chi^2}$ SE, where n is the number of measurements and χ^2 the reduced chi-square.

The shape of the distribution of elemental concentrations measured in different cells was determined by defining each measurement as a computer-generated Gaussian curve with the centroid having the value of the measurement and its width the SD. The resultant Gaussian curves were added together, normalized to the number of measurments made, and displayed (Fig. 6) on the PDP 11/05 computer (Digital Equipment Corp., Maynard, Mass.). Correlations between elemental concentrations (Fig. 7) were calculated by regression analysis.

Estimate of Errors of Measurement and of Cell-to-Cell Variability

Errors caused by statistical fluctuations follow essentially Poisson statistics and are included in the SD of the measurements. The major potential sources of systematic error are the extraneous mass added through contamination and by the carbon support film, mass loss caused by radiation damage, and miscalibration of the x-ray detector (49). Contamination in the present study has been reduced to negligible levels through the use of the cold stage. The carbon support film can add ~15% mass (as measured by x-ray continuum counts) to the overlying section, thus reducing the measured concentration by ~15%. On the other hand, mass loss of organic constituents caused by radiation damage will lead to an apparent increase in the concentration of the inorganic components by ~13% in fibrous proteins (49); hence, the systematic errors caused by, respectively, the carbon film and radiation damage would tend to cancel. For this reason, although we measured the carbon film continuum, we did not subtract it from the experimental spectra, and we consider the accuracy of the measurements to be better than 15%. Systematic errors resulting from miscalibration of the energy dispersive detector were monitored by the χ^2 value of the multiple least squares (49): by monitoring χ^2, the calibration of the energy centroid was maintained within the statistical accuracy of the measurement of the Gaussian curve (~2 eV). Hence, in the case of elements having non-overlapping peaks, the errors caused by miscalibration of the detector were definitely <5% (see Fig. 2, in reference 49). However, because of the partial overlap of the Ca-K_α- with the K K_β-peak, the absolute error introduced by even minor fluctuations in detector calibration can be somewhat greater when low concentrations of Ca are measured in the presence of high concentrations of K. This is particularly the case if the error is the result of a change in detector resolution rather than a shift in the centroid (see Fig. 2*c*, in reference 49). Thus, in the presence of 550 mmol/kg K, a 2-eV change in detector resolution could lead to an erroneous measurement of ~2 mmol/kg dry wt Ca even in a Ca-free specimen. However, a fluctuation in the detector resolution in the opposite direction would subtract an equivalent amount of Ca, and such errors in detector resolution would tend to cancel. Nevertheless, the possibility of an absolute error of this magnitude in measuring low (i.e., cytoplasmic) Ca concentrations in the presence of the physiological high K concentrations must be considered in assessing the physiological implications of such measurements.

The relationship between peak/continuum and concentration in a biological specimen is dependent upon the concentration of elements which have a higher atomic number than that of the average biological ma-

trix: for the elements in the range of Na to Ca (Z = 11–20), it is linear up to a concentration of ~1 mol/kg (49). For higher concentrations of these elements, the relationship is not linear, but can be expressed by a modified form of the quantitation equation (Eq. 5 and Fig. 5 in reference 49). For the most extreme case reported in this study, that of the nuclear composition in muscles contracted with 80 mM KCl (Table III B), the concentration of elements of higher atomic numbers (Z = 11–20) is 2.39 mol/kg with an average atomic number of ~16.7. Correction for the higher atomic number contribution to the continuum would result in concentrations 25% higher than those shown in Table III B. In all other instances, the concentrations corrected for higher atomic number contributions would vary by 15–20% from the tabulated results. More importantly, for the comparisons made in paired analyses (e.,g., between the mitochondria or nucleus and cytoplasm), the potential error resulting from differences in atomic composition is <5%.

Although we do not deal in detail with cell-to-cell variations in electrolyte content in this work, an example of the estimate of biological variability based on other techniques is given here for readers who note the range of values obtained in individual cells. In the case of cell K at a concentration of 180 mmol/kg cell H_2O (see Discussion), the range of dry wt values is from 540 to 720 mmol/kg dry wt, depending on whether the cell H_2O is 75% or 80%: values considered to be within the physiological range of cell hydration for smooth muscle. Furthermore, given the logarithmic dependence of membrane potential on the K concentration (Nernst equation), even a 17-mV range of membrane potentials could be a accompanied by a twofold difference in intracellular K. The range of membrane potentials measured in rabbit PAMV smooth muscle is 25–55 mV (within 1 SD from the mean of 40 mV, [63]). Therefore, we do not consider the cell-to-cell variations in electrolyte contents determined by electron probe analysis outside the range expected on the basis of independent measurements.

RESULTS

Structure

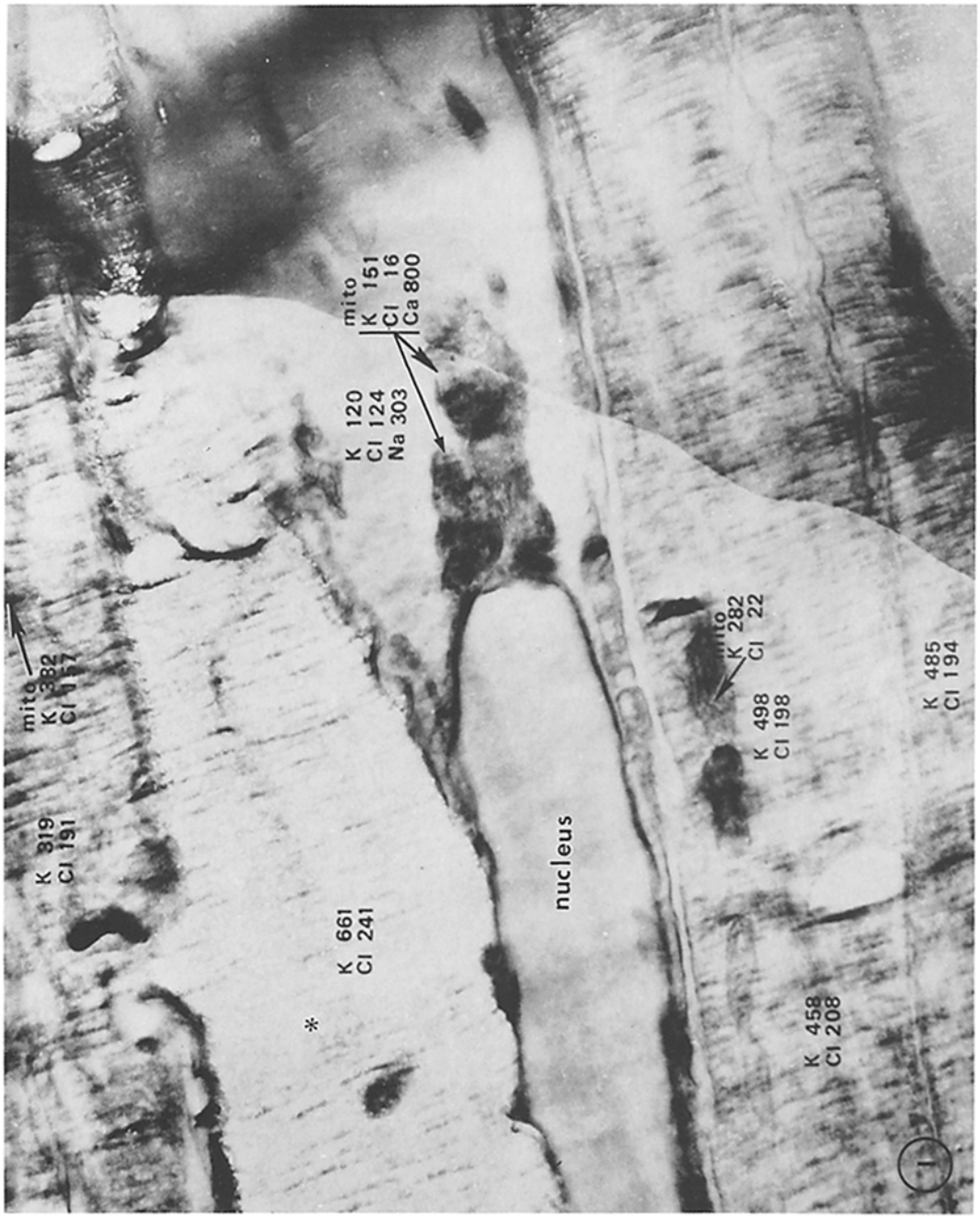
"Vitreously"[3] frozen cryosections were somewhat more difficult to obtain from smooth than from striated muscle, possibly because of slightly greater hydration of smooth muscle. Compression marks were also more prominent in smooth muscle sections, and reflect the fact that sections are removed from the knife "dry," without flotation. The electron micrographs of unstained cryosections illustrate a range from vitreous freezing (Figs. 1, 2, and 4) to moderate ice crystal formation (Fig. 3). The sections frequently showed a gradation from vitreously frozen material on the surface of the curved dome of the block (59) to ice crystals of increasing size at greater depth (Fig. 4). At the spatial resolutions required, fibers showing small (~100–200 Å) ice crystals were suitable for electron probe analysis. An occasional fiber in a fiber bundle was more electron transparent than its neighbors and contained somewhat larger and more frequent ice crystals (Fig. 1). Electron probe analysis of the light fibers suggests that this appearance was probably a result of their greater hydration, as they contained higher concentrations of elements in solution (e.g., K). Nuclei and, in the extracellular space, collagen were readily identifiable features even in vitreously frozen, unstained cryosections (Figs. 1, 2, and 4). Cell borders could also be seen in vitreously frozen sections. Mitochondria appeared as relatively electron dense and easily identifiable structures (Figs. 1, 3, and 4), although cristae were generally not visualized without vapor staining with Os (Fig. 1). Similarly, the surface vesicles of the plasma membrane could be visualized after Os vapor staining, but generally not in the unstained cryosections. Some portions of the sarcoplasmic reticulum (SR) could be identified in the unstained sections through small electron-opaque granules (Fig. 9) containing Ca (see below), but visualization of the SR membranes generally required vapor staining with Os (57). Thick and thin filaments could not be resolved in unstained cryosections, although they are readily identifiable in cryosections after negative staining (54).

Electron Probe Analysis

Cytoplasmic Composition and Sarcoplasmic Reticulum

A representative x-ray spectrum of a smooth muscle fiber (shown in Fig. 2) is illustrated in Fig. 5 and the results of cytoplasmic analyses with large (≥0.5 μm Diam) probes of normal and high KCl depolarized fibers are shown in Table II. The high cytoplasmic Cl concentration is a characteristic feature of smooth muscle spectra (see Discussion). Analysis of adjacent regions of a given fiber with 0.5-μm probes showed that, at this spatial resolution, cytoplasmic Cl was uniformly distributed within the fiber. The frequency distribution

[3] The term "vitreous" is used here to denote the absence of visible ice crystals at the electron microscope magnifications used. No inference should be drawn regarding a truly vitreous physical state of water in the specimen.


mito
K 382
Cl 157
K 319
Cl 191
K 661
Cl 241
*
K 120
Cl 124
Na 303
mito
K 151
Cl 16
Ca 800
nucleus
mito
K 282
Cl 22
K 498
Cl 198
K 458
Cl 208
K 485
Cl 194

of the cytoplasmic K concentrations was normal (not shown). In muscles that have been exposed to high KCl solutions (Table II B), the K and Cl concentrations were increased. The difference between the mean K concentrations of the KCl-treated and normal fibers (222 mmol/kg dry wt) was greater than the difference between the respective Cl concentrations (165 mmol/kg dry wt). The theoretically expected (2) gain in KCl for a fiber maintaining a constant 78% cell water volume (see Materials and Methods) is 284 mmol/kg dry wt. However, because the majority of these muscles were not paired (i.e., the resting and KCl-stimulated muscles were not obtained from the same animal), a more quantitative comparison of the changes in ion contents caused by high KCl is not warranted.

The Na concentrations showed very large fiber-to-fiber and animal-to-animal variations (Table II), but the frequency distribution was not bimodal (Fig. 6). Excluded from these tabulations were measurements made on fibers that were deemed to be damaged as shown by the presence of mitochondrial calcium granules, high cytoplasmic Na, and low cytoplasmic K (see below).

The cytoplasmic [(Na + K) − Cl] was 552 mmol/kg dry wt ± 3.2 (± SEM, n = 166). The correlation between the sum of the weighted cytoplasmic Na + K and the Cl concentration, plotted in Fig. 7, is linear and has a slope of 1.7. Cytoplasmic Na and K concentrations were uncorrelated (r = 0.03).

The contents of the SR, analyzed with small probes (Fig. 11) in KCl-depolarized fibers, were (in mmol/kg dry wt ± SEM): Na, 122 ± 16; Mg, 29 ± 6.4; P, 409 ± 10.2; S, 175 ± 6.1; Cl, 312 ± 7.3; K, 422 ± 9.6; Ca, 255 ± 7.0 (n = 14). The high Ca concentrations measured (and associated with higher than cytoplasmic P), however, may not represent the average concentration of Ca in the SR, because these structures were identified by the increased electron opacity of sequestered Ca. The Na and Cl concentrations in the SR and in the adjacent cytoplasm were not significantly different.

The normal cytoplasmic S concentrations measured reflect the fact that these analyses were made on a liquid N_2-cooled stage. During some preliminary analyses at room temperature, we had occasionally noted in both smooth and striated muscle the loss of S resulting from radiation damage (see also reference 15).

Nucleus

Nuclei and adjacent regions of the cytoplasm were analyzed with identical probe parameters. Representative x-ray spectra of a nucleus and adjacent cytoplasm are shown in Figs. 5 and 8, and the results obtained in normal and in depolarized (80 mM KCl) muscles are summarized in Table III.

The nuclear P and K concentrations are significantly higher and the Cl and S concentrations are lower than the respective cytoplasmic concentrations. The relative degrees of hydration of the two regions are not significantly different, as indicated by the comparable values of the continuum counts (last row, Table III). Nuclear Na and Ca concentrations did not differ significantly from cytoplasmic ones.

Mitochondria

MITOCHONDRIA IN NORMAL FIBERS: The composition of mitochondria, and most specifically their Ca content, was different in normal and in damaged cells. The results presented in this subsection were obtained from mitochondria in fibers having normal cytoplasmic Na and K concentrations.

Representative x-ray spectra of a mitochondrion and of the adjacent cytoplasm in the cryosection shown in Fig. 4 are shown in Fig. 8. A summary of the results of analyses of mitochondria in normal resting and in KCl-contracted nor-

FIGURE 1 Cryosection of normal rabbit PAMV smooth muscle showing the results of electron probe analysis (without error figures) imprinted in the area where the measurements were made. The cell occupying the center of the figure is damaged, as shown by the low cytoplasmic K and high Na concentrations; the mitochondrion at the nuclear pole contains Ca in the form of granules. One fiber(*) is more electron lucent than the others in this section, contains fine ice crystals, and has a relatively high K and Cl concentration suggestive of a higher degree of hydration. The Ca content of the mitochondria in the normal cells was below detection levels. Mitochondrial cristae are visible. This section was vapor stained with Os in vacuo to enhance contrast for illustration. × 37,000.

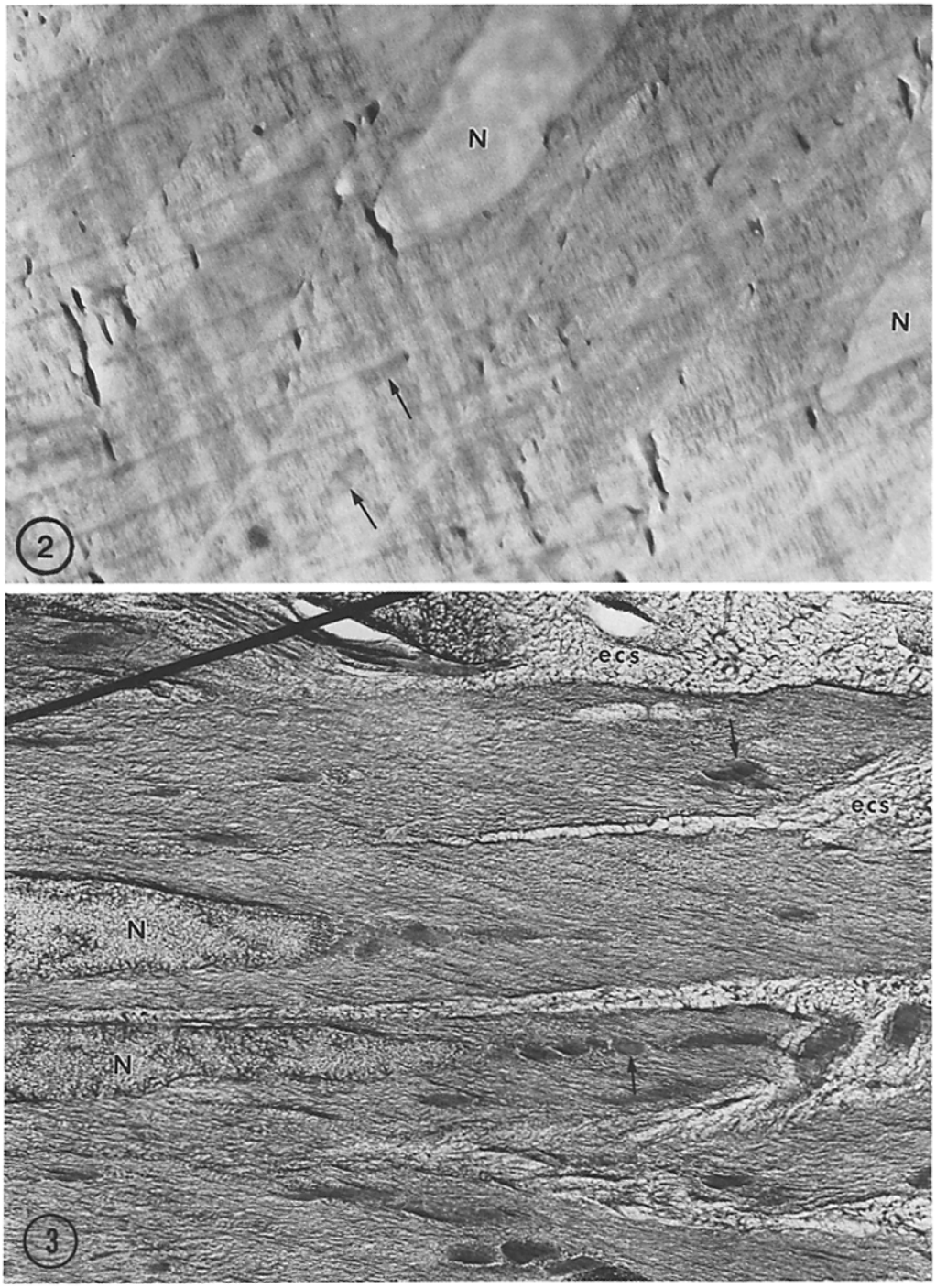


FIGURE 2 Unstained cryosection of PAMV smooth muscle showing vitreous freezing. The knife marks running diagonally from left to right are not obliterated because the section is not allowed to thaw before drying. *N*, nucleus. Arrow, mitochondrion. × 14,000.

FIGURE 3 Unstained cryosection of PAMV smooth muscle showing some increase in contrast in the presence of small ice crystals. The cell outlines, mitochondria (arrow), nucleus (*N*), and the extracellular space (*ecs*) are clearly discernible. × 14,400.

mal fibers is shown in Table IV. The most obvious feature of the mitochondrial spectrum is the relatively high P peak.

The Ca concentration in the mitochondria was low both in the resting and in the contracted muscles, and did not significantly exceed (on a dry mass basis) the Ca concentration in the adjacent regions of cytoplasm. In the KCl-contracted smooth muscles, the mitochondrial and cytoplasmic Ca concentrations were not significantly different, although both were somewhat higher than in the resting muscles. For reasons noted in Materials and Methods, we do not wish to attach undue significance to these minor differences at low Ca concentrations. We have also analyzed mitochondria in two muscles which were contracted with Na-free (K_2SO_4) solutions (see Materials and Methods) to minimize possible effects of Na-Ca exchange across the plasma membrane (1) and/or Na-induced Ca-release from mitochondria (10). The results of these analyses are shown in Table V, and also indicate the absence of mitochondrial Ca sequestration in the contracted muscles.

The concentrations of K, Na, and Cl were lower, on a dry wt basis, in the mitochondria than in the adjacent cytoplasm. However, the greater number of x-ray continuum counts obtained (with identical probe parameters) from mitochondria than from the adjacent cytoplasm indicates that the mitochondria are less hydrated (contain a relatively higher amount of dry solid/unit microvolume) than the adjacent cytoplasm. Assuming that cytoplasmic water, determined by independent methods (29), is ~78% in portal vein smooth muscle cells, the degree of hydration of the mitochondria can be computed from the ratio of x-ray continuum counts obtained from mitochondria and from the adjacent cytoplasm. Hence, subject to the assumption that mitochondrial K, Na, and Cl are in solution, the concentrations of ions in the mitochondrial water can be estimated. The ratios of these concentrations (in H_2O) in mitochondria and in adjacent cytoplasm are shown in Table IV. The values fall between 0.9 and 1.2, thus indicating that for both the cations and Cl the mitochondrial/cytoplasmic gradients are minimal. It is interesting that in the muscles contracted for 30 min with 80 m-M KCl and containing increased cytoplasmic K concentrations, the mitochondrial K content is higher than in the normal muscles incubated in the Krebs' solution, although the mitochondrial/cytoplasmic ratios are still comparable.

MITOCHONDRIA IN DAMAGED FIBERS: Mitochondria containing electron-opaque granules in unstained cryosections were found in a highly variable number of fibers from different preparations (Fig. 9). In most preparations, none of the sections contained mitochondrial granules. In some cryosections, one or several cells in a muscle bundle contained mitochondrial granules, while adjacent fibers contained none.

The mitochondrial granules contained Ca (up to 2 mol/kg dry wt) and P. A representative x-ray spectrum of a mitochondrion that contains granules is shown in Fig. 10.

The characteristic feature of cells containing mitochondrial granules was their abnormally high Na and low K content. Representative cytoplasmic concentrations in adjacent fibers, some of which contained mitochondrial calcium granules, are shown in Table VI and are taken from the same experiment illustrated in Figs. 9 and 10. The K concentrations in these high Na fibers that contained mitochondrial granules were invariably reduced from the normal (resting or KCl-treated) values. The cytoplasmic concentrations (mmol/kg dry wt ± SEM) in fibers containing mitochondrial granules were: Na, 445 ± 22; and K, 62 ± 4.1 in three fibers in muscles maintained in Krebs' solutions; the respective values in KCl-depolarized muscles were 319 ± 13.4 and 545 ± 8.2 (n = 11). Other evidence of cell damage in some of these fibers included abnormally high (15 ± 2.0, n = 11) cytoplasmic Ca concentrations. High levels of cytoplasmic Na (in conjunction with normal cytoplasmic K) were occasionally also observed in fibers containing mitochondria with a normal Ca content (see cytoplasmic Na values in Table II).

DISCUSSION

Cytoplasmic Composition, Electroneutrality, and Osmotic Equilibrium

The cytoplasmic concentration of K (611 mmol/kg dry wt ± 1.7, or 172 mmol/cell H_2O)[4] was significantly higher in vascular smooth muscle than in striated muscle (59). Flame photometric measurements of cell K in PAMV (30, 31, and, for review, see reference 29) and electrophysiological estimates in another vascular smooth muscle (26) also show K concentrations of 170–190

[4] Values are computed on the basis of 78% cytoplasmic water.

ecs
Nucleus
ecs
Nucleus
4

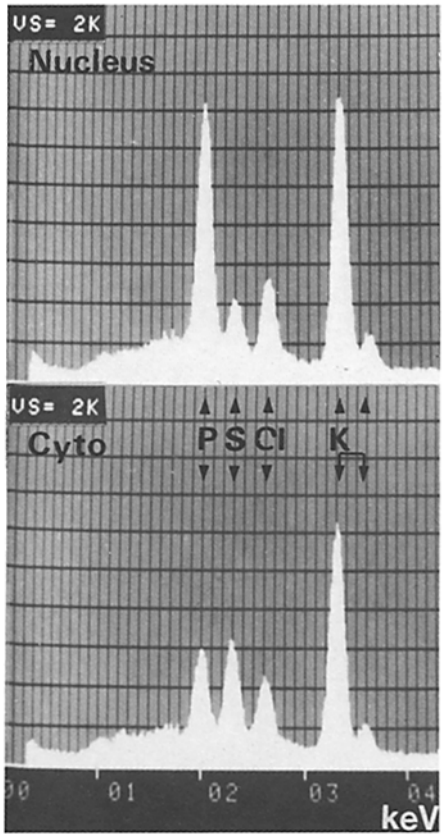


FIGURE 5 Representative x-ray spectra of nucleus and cytoplasm of the same cell shown in Fig. 2. Note that the P peak is much higher in the nucleus. The contaminating Si- and extraneous Cu L-peak have been removed by the computer program.

mmol/kg cell H_2O. The excellent agreement between the flame photometric value and the results of electron probe analysis of cryosections is particularly encouraging, because for both techniques the measurement errors are smallest for K. The count statistics and precision of the electron probe measurement are accurate to within 5%, because the cytoplasmic concentrations as well as the x-ray fluorescence yield of K is relatively high, the absorption of the potassium K x-rays by the Be window of the detector is negligible, and the principal (K_α) line is not overlapped by any other component of the physiological spectrum. The cell K values obtained with chemical measurements are also reliable, because cell K concentration is high, the extracellular concentration is low, and extracellular binding negligible (29). Hence, the results of the K measurements provide a reliable and independent estimate of the accuracy of electron probe analysis.

The Cl content (278 mmol/kg dry wt ± 1.0, or 78 mmol/cell H_2O)[4] of vascular smooth muscle was greatly in excess of that expected on the basis of a Gibbs-Donnan distribution (2), as also predicted on the basis of chemical analysis and ion flux studies of several smooth muscles (6, 30, 31, 33). The major result of the electron probe study is the demonstration that this high Cl is distributed within the cytoplasm rather than compartmentalized in organelles. The nonDonnan distribution of Cl in smooth muscle could be caused either by binding or by an active Cl pump. In cardiac and skeletal muscles and in nerve, the presence of excess (nonDonnan) Cl has been ascribed to the operation of a Cl pump (for review, see references 59 and 65), and we consider this to be the most likely mechanism in smooth muscle.

The cell Na content (167 mmol/kg dry wt, or 47 mmol/cell H_2O)[4] was relatively high in PAMV smooth muscle, and these values showed the greatest cell-to-cell variation as well as the greatest scatter among different animals (Table II). During dissection, smooth muscle cells gain NaCl and water that is subsequently extruded during incubation in physiological solutions (for review, see reference 52); the incubation times used in these experiments may not have been sufficient for ionic recovery in all the fibers. The problem of cellular Na concentration in smooth muscle has never been satisfactorily resolved with bulk chemical methods because of the large extracellular spaces and very rapid Na efflux in these tissues (e.g., 16, 17). The high Na content of blood vessels has been variously ascribed to extracellular binding or to sequestration in organelles (16; for review, see reference 29). Jones and Miller (30) recently estimated the cellular Na concentration in the

FIGURE 4 Unstained cryosection of PAMV smooth muscle bundle showing gradation from a vitreously frozen region (cell on the right) to one showing fine ice crystal formation. Muscle contracted for 30 min with high K solution before freezing. Arrowhead, collagen. Arrow, mitochondria. *ecs*, Extracellular space. × 15,400.

TABLE II

Cytoplasmic Composition of Rabbit Portal Anterior-Mesenteric Vein Smooth Muscle in Krebs' Solution

Rabbit	Date	No. fibers	K	Na	Cl	Mg	Ca	P	S
						mmol/kg dry wt			
A. Normal									
1	4-6-76	19	431 ± 3.6	205 ± 6.9	213 ± 2.3	28 ± 2.5	2.0 ± 1.3	212 ± 2.7	234 ± 2.6
2	4-20-76	20	623 ± 5.3	133 ± 7.3	211 ± 2.5	35 ± 2.7	0.4 ± 1.5	251 ± 3.1	328 ± 3.4
3	8-18-77	8	679 ± 5.8	176 ± 9.4	429 ± 4.1	38 ± 3.9	2.6 ± 1.4	218 ± 3.2	82 ± 2.2
4	9-21-77	29	766 ± 6.2	326 ± 9.4	427 ± 3.9	40 ± 3.9	0.8 ± 1.4	320 ± 3.6	155 ± 2.5
5	9-28-77	12	844 ± 7.2	156 ± 9.1	391 ± 3.9	44 ± 4.0	2.3 ± 1.5	306 ± 3.7	145 ± 2.6
6	10-5-77	11	887 ± 10.0	93 ± 12.2	295 ± 4.3	46 ± 5.4	−0.9 ± 2.1	275 ± 4.7	91 ± 3.0
7	11-1-77	44	767 ± 4.4	141 ± 5.8	337 ± 2.4	40 ± 2.7	3.6 ± 1.1	263 ± 2.4	287 ± 2.3
9	1-3-78	25	506 ± 3.6	143 ± 6.1	215 ± 2.2	35 ± 3.1	1.8 ± 1.1	201 ± 2.6	193 ± 2.2
Grand weighted mean		168	611	167	278	36	1.9	247	183
SEM			± 1.7	± 2.7	± 1.0	± 1.1	± 0.5	± 1.1	± 0.9
SD			± 192.0	± 78.5	± 94.6	± 14.3	± 5.1	± 68.3	± 101.0
B. 80 mM KCl added									
1	12-1-76	3	672 ± 18.6	93 ± 23.1	324 ± 10.8	23 ± 8.9	1.9 ± 5.0	244 ± 10.4	231 ± 9.4
2	11-15-76	7	735 ± 9.8	97 ± 10.5	352 ± 5.6	20 ± 4.2	4.4 ± 2.9	219 ± 5.0	196 ± 4.5
3	11-18-76	8	727 ± 9.4	115 ± 10.2	413 ± 6.0	19 ± 4.1	7.0 ± 2.3	188 ± 4.4	312 ± 5.3
4	12-14-76	1	898 ± 91.3	−42 ± 156.3	308 ± 40.4	80 ± 48.1	11.1 ± 23.1	358 ± 47.2	115 ± 29.4
5	4-15-77	4	886 ± 8.7	179 ± 10.3	467 ± 5.3	39 ± 4.7	9.1 ± 1.8	302 ± 4.4	113 ± 2.9
7	9-21-77	7	629 ± 10.3	304 ± 18.1	394 ± 7.3	46 ± 7.7	3.2 ± 2.7	312 ± 7.1	298 ± 6.4
8	9-28-77	13	1,205 ± 9.0	247 ± 8.4	621 ± 5.1	52 ± 3.7	3.7 ± 1.5	380 ± 3.9	174 ± 2.6
9	11-1-77	11	838 ± 9.5	273 ± 12.5	508 ± 6.6	33 ± 5.7	4.5 ± 2.2	257 ± 4.8	252 ± 4.5
10	5-16-77	6	796 ± 8.8	155 ± 12.3	368 ± 4.9	36 ± 5.2	0.6 ± 2.0	270 ± 4.5	84 ± 3.0
11	1-30-76	2	773 ± 20.6	147 ± 24.2	403 ± 11.9	15 ± 8.4	7.2 ± 5.0	397 ± 13.2	196 ± 8.9
Grand weighted mean		62	833 ± 3.4	182 ± 4.0	443 ± 2.1	33 ± 1.7	4.7 ± 0.7	281 ± 1.7	168 ± 1.3
SD			± 216.6	± 103.7	± 115.4	± 17.6	± 5.9	± 102.9	± 93.9

Weighted $\bar{x}$ ± SEM.

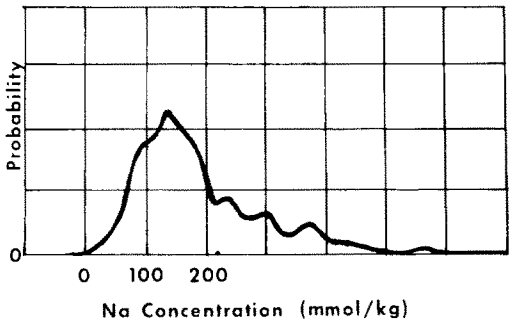


FIGURE 6 Frequency distribution of the Na concentration in 166 normal fibers.

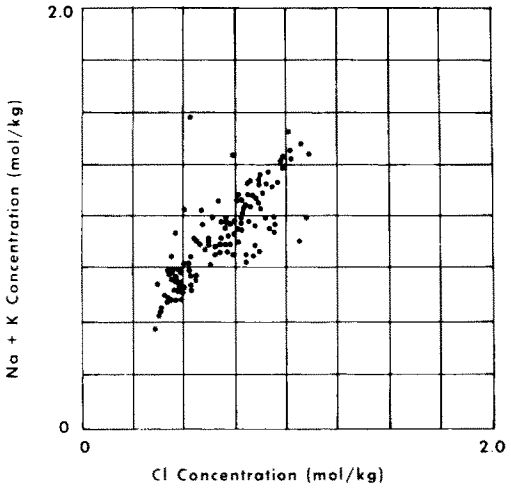


FIGURE 7 Correlation of the (Na + K) vs. Cl concentration in normal PAMV smooth muscle. The correlation coefficient $r = 0.81$; $P < 0.001$.

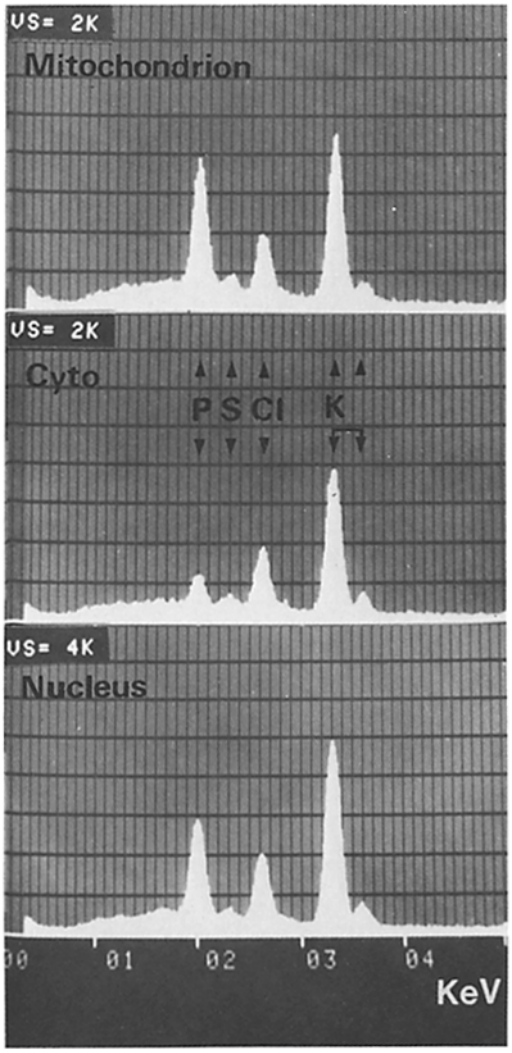


FIGURE 8 X-ray spectra of mitochondrion, cytoplasm, and nucleus of the depolarized PAMV smooth muscle fiber illustrated in Fig. 4. Extraneous peaks (Cu L and Si) have been subtracted by the computer.

rabbit PAMV by removing the extracellular component of efflux at low temperatures (1°C) in the presence of the Na, K-ATPase inhibitor ouabain. The cell [Na] of 26 mmol/cell H_2O, estimated on the basis of the efflux subsequently observed at 37°C, probably represents a lower limit, because some cellular Na efflux may also have occurred at low temperatures. In these experiments (30), the time for muscle recovery after dissection was longer (3 h) and the stretch less than in our study. This and animal-to-animal variations may also have contributed to the higher value obtained with electron probe analysis.[5] We believe that we have excluded the known sources of instrumental error of Na measurement with energy-dispersive spectrometers, because the multiple least square fitting routine that we used can easily resolve the Cu L-line (from copper grids) from the Na K-line (see Materials and Methods) and, as an independent check, the use of the Ti grids did not affect the

[5] The possibility that the inner, circular muscle of the PAMV has a significantly lower concentration of cell Na that would lead to the lower values obtained in the bulk chemical studies (30) cannot be completely excluded; however, the circular smooth muscle is usually rudimentary in the rabbit.

TABLE III

Paired Comparison of Nuclear and Cytoplasmic Composition in Rabbit Portal–Anterior Mesenteric Vein Smooth Muscles

	K	Na	Cl	Mg	Ca	P	S	Continuum
A. Normal								
Nucleus	649 ± 4.2	177 ± 6	256 ± 2.2	39 ± 2.7	2.1 ± 1.0	593 ± 4.2	95 ± 1.7	1,668 ± 11
Cytoplasm	592 ± 3.8	168 ± 5.9	282 ± 2.3	36 ± 2.6	1.6 ± 1.0	250 ± 2.4	139 ± 1.8	1,620 ± 11
Nucleus-cytoplasm (paired)	70 ± 5.8	18 ± 8.5	−25 ± 3.1	1.5 ± 3.8	0.6 ± 1.5	375 ± 5	−47 ± 2.5	3 ± 15
B. Muscles depolarized with 80 mM KCl*								
Nucleus	986 ± 9.2	189 ± 10.0	414 ± 4.5	47 ± 4.2	2.5 ± 1.7	663 ± 7.2	89 ± 2.7	2,080 ± 19
Cytoplasm	835 ± 7.8	196 ± 9.5	436 ± 4.6	44 ± 4.1	4.5 ± 1.7	269 ± 3.8	137 ± 2.8	2,193 ± 19
Nucleus-cytoplasm (paired)	128 ± 12.2	−10 ± 13.7	−27 ± 6.5	2 ± 5.9	−1.8 ± 2.4	436 ± 8.3	−50 ± 3.9	−48 ± 27

A. 28 paired analyses (nucleus and adjacent cytoplasm) obtained from six animals, mmol/kg dry wt ± SEM, except for continuum which are given as the number of counts.

B. 11 paired analyses (nucleus and adjacent cytoplasm) obtained from six animals; values are mmol/kg dry wt ± SEM, except for continuum which are given as the number of counts.

* Added to Krebs' solution.

TABLE IV

Paired Comparison of Mitochondrial and Cytoplasmic Composition in Rabbit Portal–Anterior Mesenteric Vein Smooth Muscles

	K	Na	Cl	Mg	Ca	P	S	Continuum
A. Normal								
Mitochondria	464 ± 1.7	193 ± 3.5	220 ± 1.1	38 ± 1.5	0.8 ± 0.5	571 ± 2.1	142 ± 1.0	1,789 ± 7
Cytoplasm	565 ± 2.6	246 ± 4.6	308 ± 1.7	43 ± 1.9	0.7 ± 0.7	261 ± 1.7	125 ± 1.2	1,071 ± 6
$\frac{[X_i]\ \text{mito}}{[X_i]\ \text{cyto}}$	1.2	1.1	1.1					
B. Muscles contracted with 80 mM KCl								
Mitochondria	663 ± 3.2	156 ± 0.8	303 ± 1.8	43 ± 1.9	4.6 ± 0.8	587 ± 3.0	177 ± 1.5	1,563 ± 8
Cytoplasm	871 ± 4.8	187 ± 5.4	462 ± 2.9	38 ± 2.2	4.2 ± 1.0	262 ± 2.2	161 ± 1.8	1,027 ± 7
$\frac{[X_i]\ \text{mito}}{[X_i]\ \text{cyto}}$	1.1	0.9	0.9					

A. 68 pairs of mitochondria and adjacent cytoplasm (each) from nine animals were analyzed; values are mmol/kg dry wt, except continuum which are given as the number of counts.

B. 46 pairs of mitochondria and adjacent cytoplasm from 10 animals were analyzed. $[X_i]$ = concentration in H_2O, 78% in cytoplasm, and computed as: % H_2O mito = $100 \times [1\text{-}0.22\ (Co_m/Co_{cy})]$, where Co_m and Co_{cy} are, respectively, the continuum counts originating from mitochondrion and the paired cytoplasm. N. B. Individual continuum counts, rather than the mean values shown in the table, were used to calculate X_i.

results of Na quantitation. Low Na values measured with the same electron probe instrumentation in striated muscle (59, 60) and in smooth muscle incubated in nominally Na-free solutions (present study) also indicate the absence of systematic instrumental errors which would give rise to spuriously high Na values in smooth muscle. The possibility that the Na contents measured with electron probe analysis represent the properties of adventitial, "dedifferentiated" smooth muscle fibers can also be excluded, because the cryosections analyzed contained tightly packed bundles of smooth muscle fibers known to be typical of the longitudinal muscle of the PAMV (13), and freeze-substituted blocks of the tissue remaining after cryo-ultramicrotomy also showed that the longitudinal fiber bundles had been sampled (unpublished observations). The most important result of electron probe analysis is that cell Na in smooth muscle is distributed in the cytoplasm rather than sequestered in organelles (see below). The Na and Cl concentrations in SR which contains high Ca were not significantly different from the Na and Cl concentrations in the adjacent cytoplasm. This finding, similar to that of our earlier studies on striated muscles (19, 59, 60), does not support models of Na transport based on the SR as a Na sequestration site in smooth muscle (3).

The Boyle-Conway (2) analysis of the Gibbs-

TABLE V

*Composition of Rabbit Portal-Anterior Mesenteric Vein Smooth Muscle in K_2SO_4 Solution**

	n	K	Na	Cl	Mg	Ca	P	S
Cytoplasm (large probe)‡	37	752	41	103	38	3	242	353
		± 3.5	± 4.4	± 1.2	± 2.2	± 0.9	± 1.9	± 2.1
		± 134	± 19	± 35	± 13	± 5	± 52	± 77
Cytoplasm (small probe)	16	748	52	111	42	1	239	329
		± 8.8	± 11.7	± 3.1	± 5.4	± 2.1	± 4.7	± 5.0
		± 170	± 37	± 37	± 25	± 8	± 75	± 104
Mitochondria	19	466	22	55	41	−1§	424	353
		± 4.1	± 7.9	± 1.9	± 3.8	± 1.4	± 4.3	± 3.6
		± 102	± 26	± 13	± 13	± 5	± 57	± 83
Nucleus	14	768	38	103	42	0	687	236
		± 6.6	± 7.5	± 2.2	± 3.9	± 1.6	± 6.5	± 3.1
		± 111	± 25	± 24	± 10	± 6	± 159	± 70

± SEM, ± SD.

* The results obtained with the two types of K_2SO_4 solution (see Materials and Methods) were not significantly different.

‡ >0.5 μm Diam.

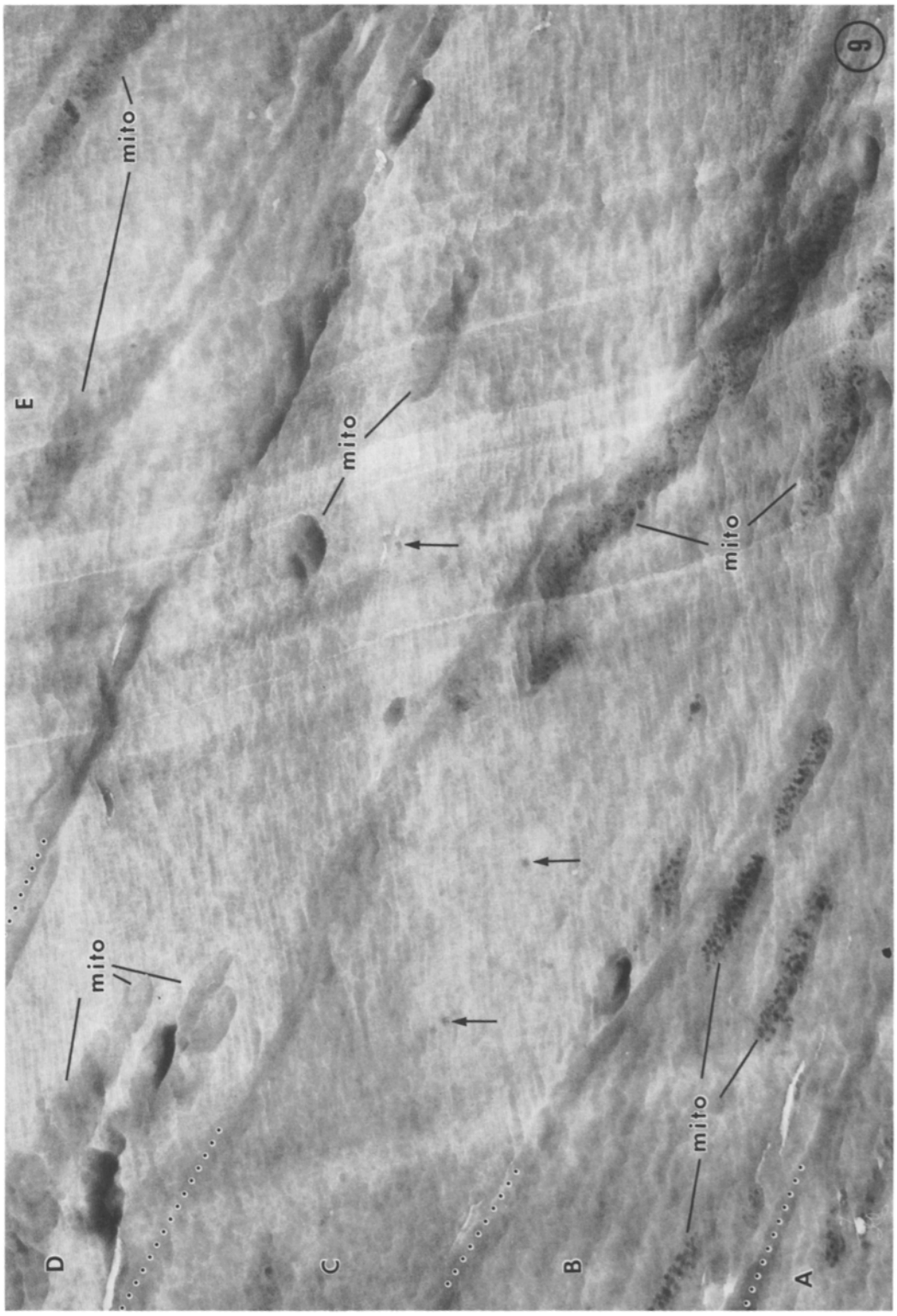
§ Negative value reflects statistical fluctuation.

Donnan equilibrium considers two quantities, η and ϵ, in explaining the ionic composition and volume of cells in salt solutions (for more detailed considerations of cell volume, see references 29, 31, and 37). η Is the concentration of intracellular nondiffusible solutes estimated, on the basis of considerations of osmotic equilibrium, as the difference between the concentration of osmotically active extracellular solutes and the known intracellular diffusible solutes. ϵ Is the concentration of intracellular nondiffusible charged solute, calculated as the difference between the known intracellular diffusible cations and anions. Our estimates are (per liter cell H_2O): η (312-Na_i-K_i-Cl_i), 14 mosmol; and ϵ (Na_i + K_i-Cl_i), 141 meq. These values are probably obtained much more accurately with electron probe analysis of Na, K, and Cl in each cell than with bulk chemical measurements of averages, although the results of the latter method (η = 60 mosmol, ϵ = 125 meq [31]) are similar. In frog striated muscles, the value of both η and ϵ is ~105 (2).

The existence of unidentified polyvalent nondiffusible anion(s) in smooth muscle is strongly suggested by the excess of ϵ over η. This conclusion would not be altered even if the cytoplasmic Na activity were lower than that of aqueous solutions (for review, see reference 7), and the activity of cell Na only that measured in flux studies (30). The discrepancy between cellular charged and total nondiffusible solute concentrations becomes even more conspicuous in comparison with striated muscles if one is to consider the much higher creatine phosphate and ATP content of the latter (see below).

The cytoplasmic P content in vascular smooth muscle was relatively high (about 250 mmol/kg dry wt); the difference between this value and the concentration in striated muscle (~300 mmol/kg dry wt [59]) is somewhat less than the differences between the creatine phosphate and ATP contents (for review, see reference 4). The high-energy phosphate contents of the rabbit *Taenia coli*, a smooth muscle that is ultrastructurally similar to the portal vein (unpublished observations), are (in mmol/kg wet wt): ATP, 1.52 ± 0.5; ADP, 0.67 ± 0.02; and creatine phosphate, 1.83 ± 0.10 (11). Thus, for a 40% extracellular space and 78% cell water, the P contributions from ATP, ADP, and creatine phosphate are, respectively: (in mmol/kg dry wt) 35, 10, and 14 for a total "accountable" P content of 59 mmol/kg dry wt smooth muscle. Subtracting this value from ϵ, some 441 meq/kg dry wt of nondiffusible solutes remain unaccounted for. In contrast, the major proportion of the nondiffusible ions in striated muscle can be accounted for by creatine phosphate and ATP (2).

The *ab initio* calculation of ϵ from the known amino acid composition of proteins is not feasible without knowledge of the *in situ* free charges (2; but c.f., references 28 and 36). However, there is considerably less myosin and approximately the same amount of actin in smooth muscle as in striated muscle (25, 41). Therefore, based on the contractile protein contents of the two muscles, a greater amount of nondiffusible negative charge remains to be accounted for in smooth than in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striated muscle. In view of the relatively high cytoplasmic P content of smooth muscle, it is possible that unidentified nondiffusible polyphosphates account for a significant proportion of ϵ.

The Mg concentration measured with electron probe analysis in the cytoplasm of rabbit PAMV smooth muscle is in good agreement with the results of bulk chemical measurements (29), which, because of the predominantly intracellular localization and slow transmembrane efflux of Mg, are considered accurate.

Nuclear Composition

There was no significant difference between the nuclear and the cytoplasmic concentrations of Na and Ca. Previous indirect studies and observations on nuclei isolated from a variety of cells (for review, see reference 7), have suggested that the nucleus may be a site of Na sequestration. However, the more recent, direct studies with ion-selective electrodes (42, 43) or electron probe analysis (14, 15, 22, 45, 59) show that in striated muscle, nerve, erythrocytes, oocytes, and epithelial cells the nucleus is not a site of Na sequestration. Our results also show that the nuclear matrix does not contain high concentrations of Ca, and, therefore, pyroantimonate precipitates in the nuclei of fixed smooth muscle (12) probably reflect nonspecific adsorption of this capture agent.

The concentration of K is higher in the nucleus than in the adjacent cytoplasm, and this cannot be accounted for by differences in hydration (and hence dissolved K), because the average nuclear and cytoplasmic dry mass contents, as measured by x-ray continuum counts, were not significantly different. The nuclear Cl content was somewhat lower than of the cytoplasm, and it is most likely that the excess nuclear K is associated with nondiffusible negative charges in the nuclear matrix. In *Chironomus* salivary gland the nuclear and cytoplasmic K activities, measured with ion-selective electrodes, are not significantly different (43).

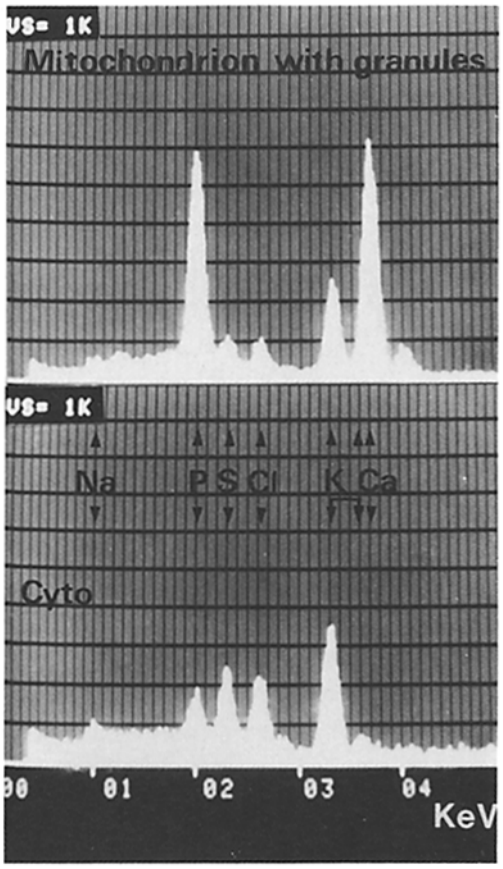

FIGURE 10 X-ray spectrum of mitochondrion containing granules and of adjacent cytoplasm. Note the high mitochondrial Ca and P and the Na peak in the cytoplasm. Extraneous peaks (Cu L and Si) subtracted by computer.

Mitochondrial Ca and Transmitochondrial Gradients

The Ca content of mitochondria in normal rabbit smooth muscle cells was low, comparable to the Ca content of mitochondria isolated from a variety of tissues (5), and in contrast to the high Ca content of the SR. Furthermore, we did not

FIGURE 9 Unstained cryosection of PAMV smooth muscle including several damaged fibers containing mitochondria with numerous Ca- and P-containing granules. Cells *A*, *B*, *C*, and *E* contain mitochondrial granules. Fiber *C* also contains Ca deposits (arrows) in the SR. In fiber *D*, the mitochondria do not contain granules, but one small deposit of Ca was present in (presumably) the SR. Fibers containing mitochondrial granules had high cytoplasmic Na and low K concentrations (see Table VI). Dots, cell borders. × 27,500.

TABLE VI

Examples of Cytoplasmic Elemental Concentrations of Rabbit Portal Vein Smooth Muscle Incubated in Krebs' + 80 mM KCl

	Na	P	Cl	K
	mmol/kg dry wt ± SD			
Cell without Ca^{++} granules	80 ± 34	249 ± 15	378 ± 17	728 ± 29
Cell with Ca^{++} granules "only" in SR	67 ± 60	198 ± 25	363 ± 29	731 ± 50
Cell with mitochondrial and SR Ca^{++} granules	554 ± 69	145 ± 22	748 ± 52	502 ± 36

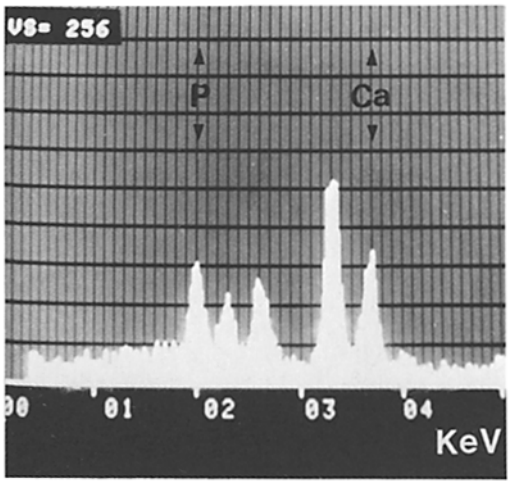

FIGURE 11 X-ray spectrum of an element of the SR in normal PAMV smooth muscle fiber. Note the large Ca and P peaks in the SR spectrum. Extraneous peaks (Cu L and Si) subtracted by computer.

find a significant transmitochondrial-cytoplasmic Ca gradient indicative of Ca uptake even after a 30-min contracture, during which the cytoplasmic free Ca is thought to be maintained at $\sim 10^{-6}$ M (25). The maximal uptake rates attainable by isolated vascular smooth muscle mitochondria are 4–12 nmol Ca/s-mg mitochondrial protein (with an apparent K_m of 17 μM), and their maximal capacity is ~1.8 mol Ca/kg mitochondrial protein (64, 68). If the apparent K_m of vascular smooth muscle mitochondria *in situ* were significantly lower, as required of an effective relaxing system (e.g., a K_m of 10^{-7} -10^{-6} M), then massive mitochondrial accumulation of Ca would be expected to take place during a 30-min contraction. It is unlikely that such massive accumulation occurred but was balanced by concomitant release (10), because the latter would lead to futile cycling and uncoupling of the mitochondria in contracting smooth muscle. Studies of smooth muscle energetics show no evidence of uncoupling of oxidative phosphorylation during contraction (for review, see references 4 and 44). Therefore, we conclude that, at least in the rabbit PAMV smooth muscle, mitochondria do not play a significant role in contractile regulation through the modulation of cytoplasmic free Ca levels. This conclusion is also supported by the relatively high apparent K_m of mitochondria isolated from smooth muscle (64).

In damaged fibers, massive mitochondrial Ca loading, in the form of granules also containing P, was readily demonstrated by electron probe analysis. The fact that the cells containing such massively Ca-loaded mitochondria are damaged can only be demonstrated through quantitation of the high cytoplasmic Na and low K in cryosections. Mitochondria Ca granules have also been found in cryosections of (damaged) cardiac muscle (62) and epithelial cells (22). Because diffusible cytoplasmic ions are lost during fixation, cell damage (e.g., during dissection) may be undetected in fixed tissues containing mitochondrial Ca granules (e.g., 55). It should also be recognized that the presence of mitochondrial Ca granules in myocardium (46) or in other tissues (48) obtained from normal animals does not necessarily indicate their presence in normal cells. In particular, the surface cells will be damaged in tissue "fragments" that are "cubed" before freezing. Because cryosections obtained from the tissue surface also contain the "best frozen" (ice crystal-free) cells to be used for electron probe analysis, such methods of tissue preparation may lead to the conclusion that mitochondrial Ca granules occur in normal tissues (see also reference 61).

The low mitochondrial Ca content of normal rabbit PAMV smooth muscle cells *in situ* is comparable to that found in normal mitochondria isolated from the same blood vessel (57), but contrasts with the high endogenous Ca content of

mitochondria isolated from presumably normal bovine blood vessels. Furthermore, the Ca and Mg contents are even higher in mitochondria isolated from atherosclerotic bovine arteries (57). Therefore, the possibility must still be considered that mitochondrial Ca may vary in smooth muscle as a function of species, disease, and other factors.

There was no significant Na, K, or Cl gradient between mitochondria and cytoplasm, contrary to what would be expected on the basis of a large inside negative mitochondrial potential implied by the chemiosmotic hypothesis of oxidative phosphorylation (39). Examining the assumptions involved in our estimates of transmitochondrial distribution, we note that any binding of Na and K (assumed by us to be dissolved in mitochondrial H_2O) by the mitochondrial matrix would result in a more positive calculated mitochondrial emf_{Na} and emf_K and, hence, reinforce our conclusions. We recognize that the ions considered need not follow a Donnan distribution if the mitochondrial membrane is impermeable to them. However, even considering a very low mitochondrial permeability to K and Na, the values measured in resting muscle presumably represent an equilibrium state that, barring other transport mechanisms, may be expected to reflect a passive distribution. Furthermore, Cl appears to follow a Donnan distribution in isolated mitochondria (66), and the *in situ* distribution of Cl found by us is also inconsistent with a large transmembrane transmitochondrial potential gradient. Our conclusions about the transmitochondrial Donnan (Na, K, Cl) potential would also not be altered by a relatively large error in electron probe quantitation (e.g., 25%) in view of the logarithmic concentration dependence of the Donnan potential. Therefore, our findings are in agreement with the recent microelectrode studies (38) that did not show large negative potentials in isolated giant mitochondria. The higher mitochondrial K content of muscles exposed to high KCl suggests that mitochondria *in situ* can accumulate K, possibly through an energized transport mechanism (32).

Electron Probe Analysis: Prospects in Cell Biology and Technical Considerations

We consider that the results observed in this study continue to indicate the value and further potential of electron probe analysis in biology. The *in situ* quantitation of the composition of organelles, correlated with the cytoplasmic composition of the individual cell, is not attainable directly by any other method. It is reassuring that reliable, independent measurements of cell K and Mg show excellent agreement with the results of electron probe analysis.

A technical consideration related to electron probe analysis of dried cryosections is the necessity of considering the relative states of in vivo hydration of different domains. Identical concentrations of a given element in water will give rise to different concentrations on a dry weight basis as a function of the state of hydration of some subcellular domain. However, the relative degrees of hydration in different regions of a dried section can be determined by measuring the continuum counts. For example, the lower cytoplasmic in vivo water content (higher mass density in the dry state) of mitochondria was reflected in the x-ray continuum counts and the greater scattering of electrons (electron opacity). The validity of using continuum counts as a measure of hydration (in vivo) is shown by the good agreement between the calculated mitochondrial water (63%) and that measured in isolated mitochondria (66%) (67). Therefore, x-ray continuum measurements can be used in freeze-dried cryosections to correct for the different states of hydration of organelles. In more extreme instances of large aqueous domains, such as the lumen of renal tubules or capillaries, electron probe analysis of frozen hydrate tissues may be required (21, 34, 35, 47).

The most recent advance in biological electron probe analysis is the use of high brightness field emission sources to obtain x-ray maps (51) from ultrathin cryosections. In addition to providing a visual display of differences in cell composition, the information contained in computer-stored x-ray maps can be further refined with image processing techniques. We expect that this approach will find major applications in biology.

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