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Calcium and sodium distribution and movements in smooth muscle

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Summary. Electron probe microanalysis (EPMA) has been used to study the subcellular distribution of Ca, Na, K, Cl, and Mg in smooth muscle. The EPMA results indicate that the sarcoplasmic reticulum (SR) is the major *intracellular* source and sink of activator Ca: norepinephrine decreases the Ca content of the junctional SR in portal vein smooth muscle. Mitochondria do not play a significant role in regulating cytoplasmic free Ca^{2+}, but mitochondrial Ca content can be altered to a degree compatible with suggestions that fluctuations in matrix Ca contribute to the control of mitochondrial metabolism. The rise in *total* cytoplasmic Ca during a maintained, maximal contraction is very much greater than the rise in free Ca^{2+}, and is probably in excess of the known binding sites available on calmodulin and myosin. Cell Ca is not increased in normal cells that are Na-loaded. The non-Donnan distribution of Cl is not due to compartmentalization, but reflects high cytoplasmic Cl. Na-loading of smooth muscle in K-free solutions is temperature dependent, and may exhibit cellular heterogeneity undetected by conventional techniques. The total cell Mg is equivalent to approximately 12 mM, and less than 50% of it can be accounted for by binding to ATP and to actin. Mitochondrial monovalent cations in smooth muscle are relatively rapidly exchangeable.
Key words. Ca, Mg, Na; electron probe analysis; mitochondria; sarcoplasmic reticulum; inositol trisphosphate.

Introduction

The importance of calcium and sodium in smooth muscle function has been overshadowed only by the difficulty of determining, by conventional methods, the distribution and movements of these ions. Large extracellular spaces and binding sites and the uncertainties of identifying the number and anatomical site of kinetic compartments are only some of the problems facing physiologists attempting to measure cellular sodium and to determine the distribution of cellular calcium (for review, see Jones[28] and Brading[6]). Electron optical techniques now permit the localization and quantitation of these elements in ultrathin cryosections of rapidly frozen tissues (for review, see Somlyo[48], Hall[24]). We shall summarize here the results of some studies of Ca, Na and other electrolytes in smooth muscle with these methods and functional implications concerning excitation-contraction coupling.

Methods

The methods used for rapid freezing and cryoultramicrotomy[33,53] and for quantitative electron probe analysis[4,5,34] have been described in detail, as have the physiological experiments showing contraction of smooth muscle in Ca^{2+}-free solutions[4].

Calcium in sarcoplasmic reticulum and Ca release

The rise in cytoplasmic Ca^{2+} that activates smooth muscle contraction[21] (for review, see Hartshorne[25]) can be triggered electrically by action potentials[8] (for review, see Kuriyama[36] and Johansson[27]) and graded depolarization, and/or by a mechanism independent of changes in membrane potential, pharmacomechanical coupling[11,20,26,54]. Some forms of activation are associated with Ca^{2+} influx

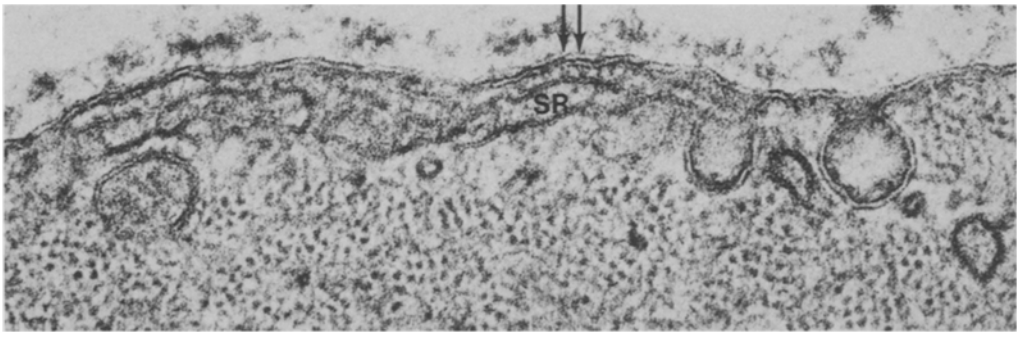

Figure 1. Transverse section from the main pulmonary artery of the rabbit showing periodic surface couplings (arrows) crossing the junctional gap between junctional SR and plasma membrane. The tissue was fixed in 2% OsO_4 followed by 2% tannic acid and then uranyl acetate. × 124,000. Somlyo[51], J. Cell Biol. *80* (1979) 743–750.

(for review, see Jones[28]), but smooth muscle can also be activated in the virtual absence of extracellular Ca^{2+} [26,38,47]. Central questions raised by these observations to be addressed here are the anatomical site of the intracellular activator Ca and the amount of *total* (as opposed to free) Ca required for activation, and how these two quantities relate to each other. In view of the possibility[7,44] (for review, see Blaustein[3] and Van Breemen[57]) that Na, in addition to its role in determining membrane electrical properties, may also control cellular Ca and tension through Na/Ca exchange, we describe some direct measurements of total cytoplasmic Na and its effect on cellular Ca.

The presence of sarcoplasmic reticulum in smooth muscle[47] (for review, see Somlyo[52] and Gabella[23]) and the demonstration that the SR could accumulate divalent cations in situ[55] established that the SR could serve as an intracellular calcium sink in smooth muscle. This conclusion was further strengthened with improved methods for isolating SR membranes from smooth muscle[41] (for review, see Sloane[45]) and by electron probe analysis of smooth muscles permeabilized with saponin[50] (for review,

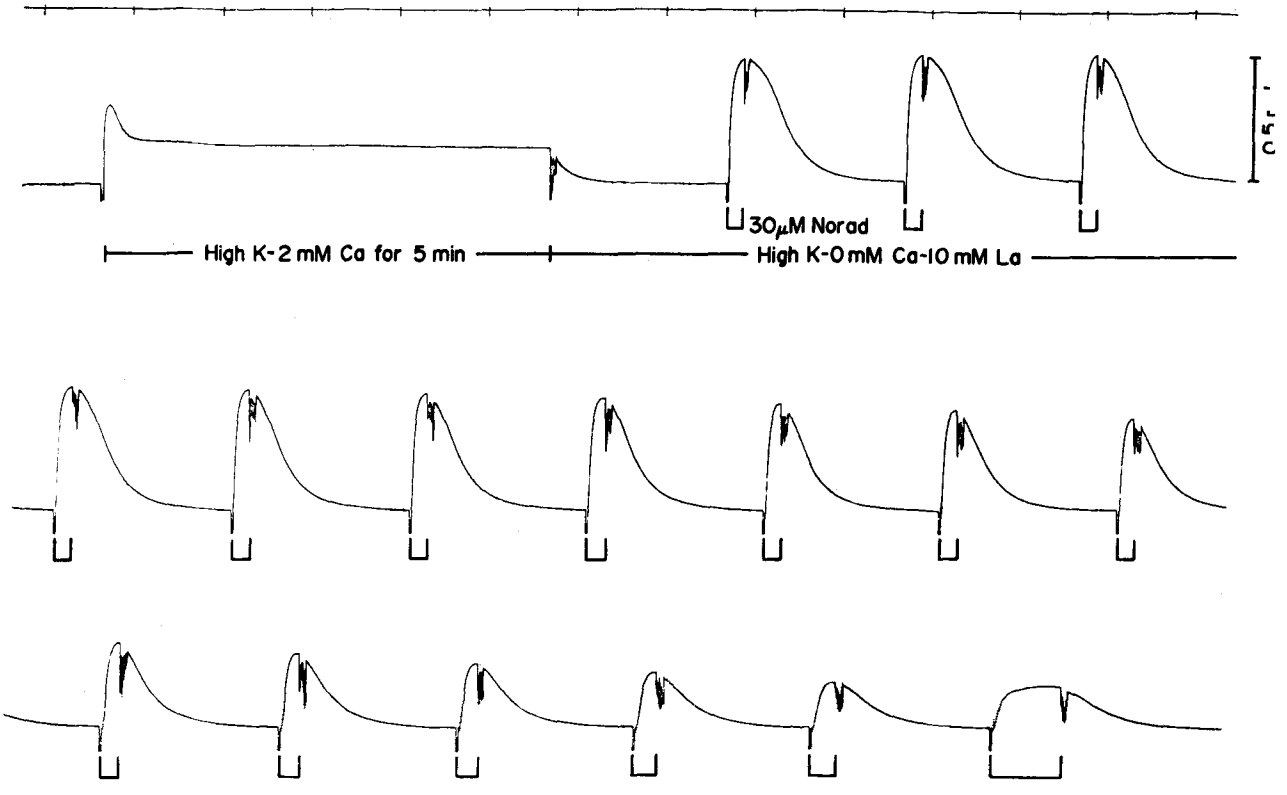

Figure 2. Repeated contractions induced by norepinephrine in Ca-free, 10 mM La^{3+} solution. The longitudinal muscle of guinea pig portal vein was first contracted by high K^+, 2 mM Ca^{2+} solution for 5 min, and then relaxed by removal of Ca^{2+} and simultaneous addition of 10 mM La^{3+}. The fibers were contracted by 30 μM norepinephrine. The drug was then rapidly removed, as soon as the contraction reached its peak. Several interrupted contractions induced by the repetitive application of norepinephrine every 2 min were larger than the K contraction in the presence of 2 mM Ca^{2+}. Time scale: 1-min interval. From Bond et al.[4], J. Physiol., Lond. *355* (1984) 677–695.

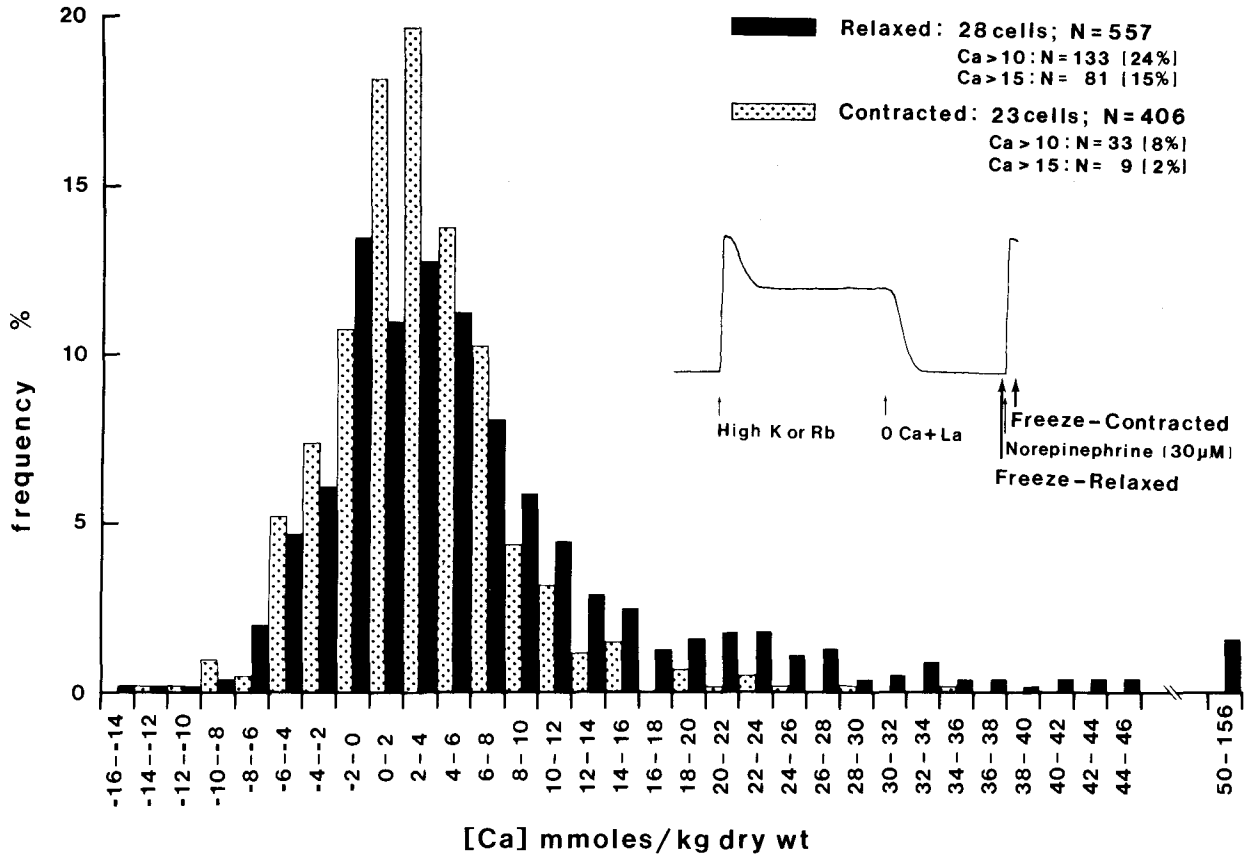

Figure 3. Histogram showing the distribution of Ca concentrations measured with small diameter (75 nm) probe analyses of the cell peripheries in the relaxed and contracted guinea pig portal veins. The tension trace and the freezing protocol for the relaxed and contracted states are shown in the inset. Negative values reflect statistical fluctuations that are relatively large. In order to make the large number of measurements required, spectra were acquired for only 100 sec each and the SD of the earlier (individual) measurements reached 3.9 mmol/kg dry wt. From Bond et al.[4], J. Physiol. Lond. *355* (1984) 677–695.

see Johansson[27]). That Ca could also be released from the SR was also supported by the fact that caffeine mobilized Ca from the same compartment as physiological transmitters, and this compartment accumulated Ca in the presence of Mg-ATP[17,19,26] (for review, see Bond[4] and Johannsson[27]). Most recently, we have shown with electron probe X-ray microanalysis (EPMA) that norepinephrine releases Ca from the SR in smooth muscle[4]. The structural feasibility of calcium release from the SR by stimuli (impermeable transmitters and action potential) acting on the surface membrane, was shown by the presence of peripheral elements of the SR (junctional SR) connected to the plasma membrane by quasi-periodic electron densities[47]. The use of tannic acid to improve structural preservation[51] accentuated the similarities between the fine structure of these surface couplings in smooth muscle (fig. 1) and the triads in striated muscle. The location of junctional SR in a relatively circumscribed subsarcolemmal region permitted the measurement of the Ca concentration in the SR in smooth muscles that were rapidly frozen either relaxed or at the peak of a contraction induced with 30 μM norepinephrine[4]. These experiments, performed in conjunction with physiological studies, demonstrate the pharmacomechanical release of Ca from the SR *in the absence of* Ca^{2+} *influx*. Contractions of guinea pig portal vein smooth muscle in 0-Ca^{2+}, 10 mM La^{3+}, high K solution are shown in figure 2. The repeated maximal and near maximal contractions in the absence of extracellular Ca^{2+} indicate the recycling of sufficient stores of intracellular Ca to evoke a maximal contraction in this smooth muscle. Only a single contraction could be produced[4], as in previous studies[12,18], when norepinephrine was allowed to remain in contact with the tissue throughout the period of relaxation.

EPMA of smooth muscles frozen in the relaxed state showed that the frequency of 'hot spots' containing high concentrations of Ca (28 mmol/kg dry wt ± 2.8 SEM) was approximately 3–4/cell periphery: the same as the frequency of junctional SR tubules in conventionally fixed preparations. The number of these 'hot spots' was significantly reduced in the smooth muscles frozen at the peak of the norepinephrine induced contraction (fig. 3). The regions containing high Ca were further identified as junctional SR in rapidly frozen and freeze-substituted muscles[4].

Total cytoplasmic calcium in smooth muscle cells

The increase in cytoplasmic calcium during a *maintained,* maximal contraction was determined in rapidly frozen *rabbit* PAMV smooth muscle preparations that had been

contracted with high K solutions containing 30 μM norepinephrine and maintained maximal tension for 30–40 min prior to freezing[5]. The control, resting preparations were in normal, Na-containing Krebs solution to which isoproterenol (0.2 μg/ml) was added 2 min prior to freezing, to inhibit spontaneous activity. The cytoplasm selected for analysis with small (approximately 100–200 nm diameter) probes was in areas of the cell cross-section distant from both the junctional SR and the SR associated with mitochondria, in order to avoid possible contamination of the cytoplasmic X-ray spectra with Ca in (nonvisualized) SR.

Cytoplasmic Ca increased by 1.0 mmol/kg dry wt ± 0.22 SEM (from 0.8 ± 0.15 to 1.8 ± 0.16) during a maintained maximal contraction[5]. This is equivalent to a rise of approximately 235 μM Ca in cell H_2O (81% [31]), and is clearly much more than the free cytoplasmic Ca^{2+}. The latter is certainly less than 10 μM and probably not more than 5 μM even during a maintained maximal contraction[50]. The measured increase in total Ca^{2+} is probably also in excess of the Ca-binding sites available on calmodulin and on myosin, suggesting the presence of other high affinity, Ca-binding proteins in smooth muscle. The increase in total cytoplasmic Ca measured in these studies is thought to represent an upper bound, or *sufficient* Ca, for a maximal contraction, as transient and/or submaximal contractions may be activated through a smaller rise in total cytoplasmic Ca. Even the large increase in cytoplasmic Ca observed during the maintained maximal contraction could be provided by a release of approximately 45 mmol Ca/kg dry wt from the SR in a smooth muscle in which the SR (containing this calcium) comprised 2% of the cell volume. The calcium release required to activate a submaximal twitch during an action potential is probably less. Therefore, we conclude that the SR contains sufficient Ca for activation of twitch contractions evoked by action potentials.

The mechanism of calcium release through *pharmacomechanical coupling* from the SR may be mediated by *inositol 1,4,5-trisphosphate* ($InsP_3$) liberated through transmitter-stimulated polyphosphatidyl inositol hydrolysis[2a, 2b, 52a, 55a]. Therefore, not only is it established that calcium-induced calcium release (through influx of extracellular calcium) is not required for drug-induced calcium release from the SR, but the probable mediator ($InsP_3$) has now also been identified. However, the mechanism of *electromechanical* calcium release from the SR, presumably involved in triggering twitch contractions, is still not known, as depolarization (at least with high potassium) does not increase phosphatidyl inositol turnover [2a, 2b].

Mitochondrial Ca and Na

There is now compelling evidence to show that mitochondria do *not* contribute to the physiological regulation of cytoplasmic Ca^{2+} during the contraction-relaxation cycle in smooth or in striated muscle. It is probable, in fact, that even in nonmuscle cells the endoplasmic reticulum is the main intracellular organelle regulating cell Ca^{2+} [39,46]. The apparent K_m for Ca^{2+} uptake of mitochondria isolated from vascular smooth muscle, in the presence of Mg^{2+}, is higher (17 μM[56]) than is required for regulating cytoplasmic free Ca^{2+} in the probable physiological range from 50 nM to 5 μM. Furthermore, no measurable increase in mitochondrial Ca has been observed with electron probe analysis of smooth muscles contracted for 30 min with high K alone[49] or together with 30 μM norepinephrine[5].

Massive increases (up to 2 mol/kg dry wt) in mitochondrial Ca (granules) have been observed with EPMA in damaged cells in which cytoplasmic Ca rose to abnormally high levels[49] and on exposure to 10 μM cytoplasmic Ca^{2+} in cells permeabilized with saponin[50]. In 'light cells' found in smooth muscle Na-loaded in the cold[31] mitochondrial Ca was somewhat increased (39–43 mmol/kg), but these mitochondria were not massively loaded, perhaps because of the loss of ATP and soluble respiratory energy sources.

A more recently observed and, perhaps, more interesting reduction in mitochondrial Ca concentration was found in smooth muscles exposed to Ca-free (16.2 mM free Mg^{2+}) solution (Table 1). In Na-loaded (at 37 °C) smooth muscle, there was a smaller reduction in mitochondrial Ca associated with the very large increase in cytoplasmic and mitochondrial Na. These observations, while still somewhat preliminary, are compatible with the existence of small fluctuations in mitochondrial Ca, of a magnitude that may regulate mitochondrial metabolism[16]. The reduction in mitochondrial Ca in Na-loaded smooth muscle was less (statistically not significant from relaxed) than in smooth muscle placed in Ca-free solutions (table 1). The relatively modest effect of even these massive increases in cytoplasmic and mitochondrial Na is compatible with studies in which Na-induced Ca release could not be demonstrated in mitochondrial isolated from uterine and ileal smooth muscle[14].

Table 1. The effect of Ca-free solutions or Na-loading on mitochondrial Ca in rabbit portal vein smooth muscle (mmol/kg mitochondrial dry wt ± SEM)

	n	Ca	Na
Normal, relaxed**	50	1.6 ± 0.2	153 ± 11.0
Normal, maximally contracted**	46	2.3 ± 0.3	202 ± 12.6
Na-loaded 37 °C, 3 h	62	1.1 ± 0.3	724 ± 19.8
Ca^{2+}-free 6.2 mM free Mg**	34	0.0 ± 0.2*	83 ± 7.6

* Significantly different ($p < 0.01$) from normal (relaxed and contracted).
** From Bond et al.[5].

Sodium

The variability of cellular Na measured with conventional methods has been ascribed to difficulties in estimating the free and bound extracellular components and to increased cellular Na due to tissue handling during dissection (for review, see Jones[28]). Methods devised to minimize extracellular contributions to Na flux measurements include the washout of extracellular Na with cold, Na-free (Li-substituted) solutions[1,22] or compartmental analysis of the temperature sensitive (cellular) component of Na radioisotope efflux[30]. The time resolution of these methods is often insufficient to evaluate fast, temperature-sensitive components of cellular efflux occurring during the first 5 min.

EPMA measurements of cell Na are not subject to extracellular contributions, as the electron probe is placed, under direct vision, on any desired region of the cytoplasm or on an organelle. Even with this method, considerable variability in cell Na has been observed in some smooth muscles. For example, the distribution of cytoplasmic Na concentrations in rabbit PAMV varies over an approximately 5-fold range[31]. Furthermore, this variation could not be related to either the extent of stretch prior to freezing or to the duration of incubation (beyond 2 h[31]). Neither in these nor in previous[49] EPMA studies was there any evidence of compartmentalization of Na in nuclei or in mitochondria.

In contrast to the variable, but frequently high (46 mM/l cell H_2O) concentration of Na in normal rabbit PAMV, the Na content of the guinea pig taenia coli was relatively low (19 mM/l cell H_2O)[31]. The Na content of rat bladder smooth muscle (22 mmol Na/l cell H_2O, assuming 81% hydration), determined with EPMA, is also relatively low.

Washout at 2 °C in Na-free (Li-substituted) solutions caused a reduction in the Na content of normal rabbit PAMV: $[Na]_i$ in the Li washed smooth muscles was 16 mmol/l cell H_2O. Furthermore, when rabbit PAMV and guinea pig taenia coli were Na-loaded for 3–4 h in cold, K-free solutions, subsequent washout with cold, Na-free (Li) solutions caused marked Na loss (from a 574 mmol/kg dry wt to 169 mmol/kg dry wt in rabbit portal vein[31]. In contrast, in normal guinea pig taenia coli, Li-wash did not cause significant reduction of cell Na.

Loss of Na from Na-loaded cells was most marked, although not limited to, a group of light cells that could also be distinguished by their abnormally low P, Mg and high Ca contents. During washout with cold Na-free (Li) solution, these light cells, thought to represent a particularly permeable cell population, lost their entire Na content. However, such cells could still be distinguished after Li washout due to their low cytoplasmic Mg, K and P content, as well as by the near absence of cytoplasmic Na.

The effect of temperature on Na-loading and washout

The temperature at which smooth muscles are Na-loaded appears to have a marked effect on the extent of Na-loading and K loss, as well as on the effect of washout in cold, Na-free (Li) solutions. Although these results are still preliminary, we present them here, because EPMA may not be readily available to a sufficient number of laboratories, and the observations are not readily detectable by other methods.

In rabbit PAMV placed in K-free Krebs solution for 3 h at 37 °C (fig. 4B), Na loading and K loss was much more uniform than after Na-loading at 2 °C (fig. 4A and Junker et al.[31]. The average K concentration in the cells loaded at 37 °C was 4.8 mmol/kg dry wt ± 0.6 SEM (n = 75), reflecting near total exchange for the Na gained, while in the cells loaded at low temperature[31] K was 356 mmol/kg dry wt + 27.7 SEM (n = 81). An interesting, incidental observation was the parallel reduction in mitochondrial K content (4.3 mmol/kg dry wt ± 0.41 SEM, n = 46) in the Na-loaded (at 37 °C) cells during the 3 h. In other experiments[4], we found that after 15 min incubation of guinea pig portal vein smooth muscle at 25 °C (in 147.5 mmol Rb^+, 0-K^+ solution) 27% of the mitochondrial K had exchanged with Rb (or 192 ± 12.7 mmol Rb/kg dry wt, n = 64), paralleling the 29% cytoplasmic exchange of Rb^+ for K^+. The results contrast with the very slow loss of K from isolated (liver) mitochondria[43], and indicate the presence of relatively rapid in situ exchange mechanisms for transporting mitochondrial monovalent cations that was also demonstrated by the parallel increase in mitochondrial and cytoplasmic K in response to high K solution[49]. This relatively rapid exchange may occur via an energy-dependent exchange mechanism[49], since the K permeability of mitochondria in situ appears to be low, as indicated by the valinomycin induced K uptake and swelling of mitochondria in intact skeletal muscle fibers[35].

The effect of Na-free, (Li) washout on the cells Na-loaded at 37 °C differed quantitatively from its effect after loading at low temperature. A high proportion of the large Na-load (fig. 4B; Na approximately 1100 mmol/kg dry wt) gained at 37 °C was retained during cold Li-washout. Occasional 'light cells' were also found after Na-loading at 37 °C, although it is our impression (still to be quantitated) that they were less common than in cells loaded at 2 °C.

The most likely interpretation of the effects of temperature on Na loading and subsequent Li-washout is that membrane permeability to monovalent cations is also reduced at low temperatures, as suggested by electrophysiological studies[32]. This may explain the lesser exchange of Na for K at 2 °C than at 37 °C. The cause of the apparently (in preliminary studies) more marked Na-washout from the cells loaded at low than at high temperatures remains to be established. One possibility is that the plasma membranes of cells loaded in the cold are more fragile and, therefore, become hyperpermeable (rupture?) as cells are massively Na-loaded. A second possible mechanism is that cells are metabolically depleted in the cold (slow ATP breakdown without resynthesis) and this causes an increase in membrane permeability[13]. Experiments are now in progress to clarify the mechanisms involved.

The Na-loaded (at 37 °C) cells that appeared normal (not 'light cells') in electron micrographs of cryosections had gained Na in amounts equivalent to the K loss: the cyto-

Table 2. Cytoplasmic and cellular Ca in normal (relaxed and contracted) and in Na-loaded rabbit portal vein smooth muscle (mmol/kg dry wt ± SEM)

	n	Cytoplasmic Ca	n	Cell Ca
Normal, relaxed†	262	0.8 ± 0.15	68	1.3 ± 0.31
Normal, contracted 30 min†	296	1.8 ± 0.16*	93	3.2 ± 0.27***
Na-loaded, 37 °C 3 h	155	0.5 ± 0.10**	58	1.1 ± 0.21
Ca^{2+}-free 6.2 mM free Mg^{2+}	110	0.6 ± 0.14	51	0.4 ± 0.20

Measurements of cytoplasmic Ca were made with 0.1–0.2 µm diameter probes and of cell Ca with 1.0–1.5 µm diameter probes; the latter include contributions from both cytoplasm and sarcoplasmic reticulum.

* Significantly different ($p < 0.0005$) from relaxed, Na-loaded and Ca-free cytoplasmic Ca.

** Significantly different ($p < 0.0005$) from normal (relaxed and contracted) and from Ca-free ($p < 0.02$) cytoplasmic Ca.

*** Significantly different ($p < 0.0005$) from relaxed, Na-loaded and Ca-free cell Ca.

† From Bond et al.[5].

plasmic Na + K remained constant and cytoplasmic Ca was *not* increased. Therefore, volume control is maintained at least over a 3-h interval, in spite of inhibition of the Na-pump, in agreement with previous observations showing the absence of significant cell swelling in smooth muscles[15,29,42] and other cells[37] inhibited with ouabain.

Cellular and cytoplasmic Ca in Na-loaded cells

To determine whether the abolition of the inward Na gradient increases cell Ca[3,57] (as opposed to cytoplasmic Ca), we measured Ca in Na-loaded (at 37 °C) cells with large diameter (1–1.5 μm) electron probes that include regions of both sarcoplasmic reticulum and cytoplasm. These measurements, therefore, parallel total cellular Ca, although they somewhat underestimate it, because they exclude a proportion of the junctional SR that is near the plasma membrane[5]. The concentrations shown in table 2 exclude measurements made on 'light cells', and indicate that cellular Ca is not increased. The Ca-content of the 'light cells' was markedly increased, but this very large increase (27.5 ± 1.66 mmol/kg dry wt; n = 8) clearly represents a pathological level and, as indicated above, was associated with a marked reduction in cell P. Our studies (present study; Junker et al.[31]) like observations with other techniques and different smooth muscles[7,40] failed to show a major, physiological role of Na/Ca exchange in the control of cellular Ca and contraction in these smooth muscles. Furthermore, the presence of hyper-

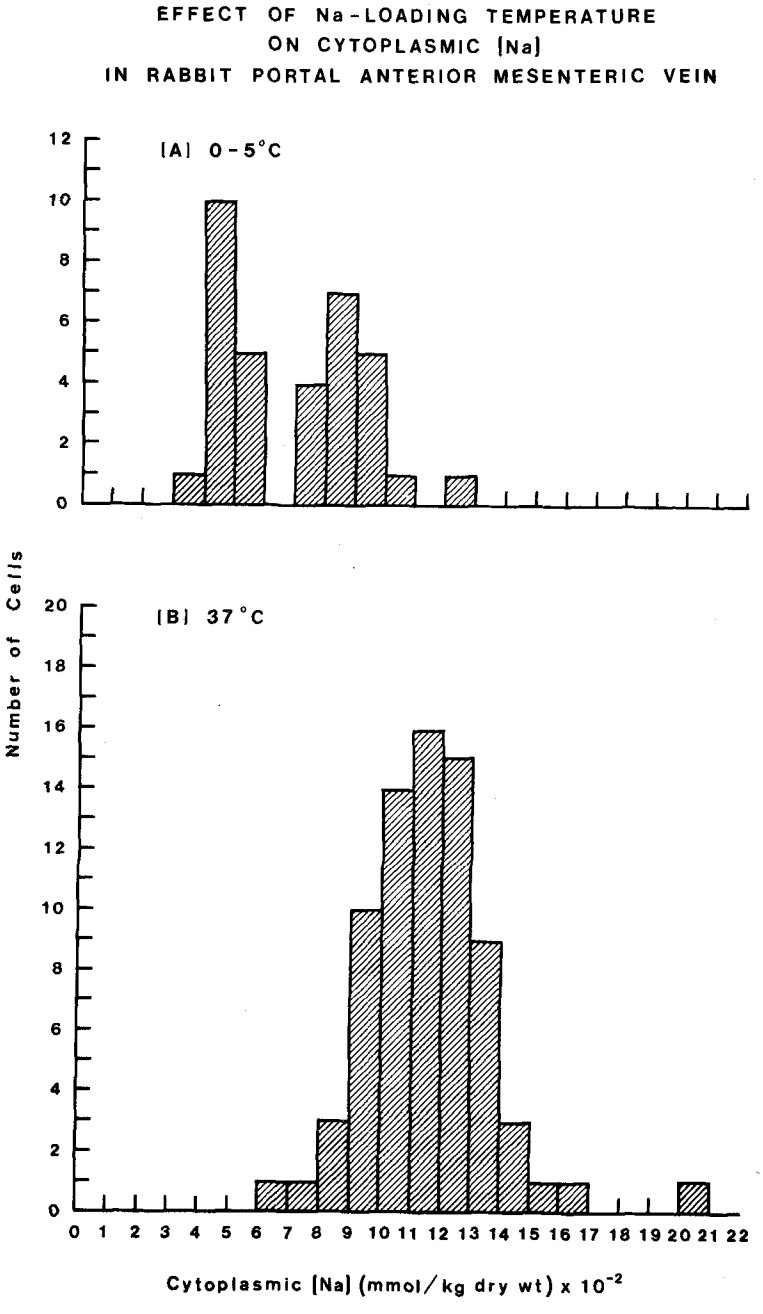

Figure 4. Frequency distribution of cytoplasmic Na concentrations in smooth muscle cells of rabbit portal anterior mesenteric vein after 3 h incubation in K-free, Na-loading Krebs solution (A) in the cold (from Junker et al.[31]) or (B) at 37 °C. Measurements were obtained from both dark and light cells.

permeable 'light cells' also containing abnormally high Ca concentrations severely complicates the assessment of the effect of Na-loading on cell Ca content with bulk methods.

The cytoplasmic concentration of Ca in portal veins Na-loaded at 37 °C was also measured with small probes that minimize the contribution of the Ca in the SR[5]: it was 0.5 ± 0.14 SEM (n = 155). The difference between this value and the 0.8 mmol/kg dry cytoplasm found with identical techniques in relaxed smooth muscle is not statistically significant, and is similar to the 'tightly bound' cytoplasmic Ca (0.6 ± 0.14 SEM mmol/kg dry wt; n = 110) in portal vein incubated in Ca-free solutions[5].

Cl and Mg

An advantage of energy-dispersive EPMA is that it simultaneously quantitates all the normally occurring cellular elements from atomic number 11 (Na) and higher. Therefore, it was evident upon EPMA[49] that Cl is uniformly distributed throughout the cytoplasm in smooth muscle, demonstrating that the 'excess' Cl efflux indicative of non-Donnan distribution of Cl in smooth muscle[10] is not due to compartmentalization in organelles. Studies with Cl-selective electrodes further showed that the cytoplasmic activity of Cl^- was also high and consistent with the results of EPMA[2]. Active Cl^- transport (Cl^- pump) associated with non-Donnan distribution of cytoplasmic Cl^- is certainly not unique to smooth muscle, but has been observed in a variety of cell systems.

Cytoplasmic Mg measured with EPMA in rabbit PAMV[5,31,49] and guinea pig taenia coli incubated in normal Krebs solution is approximately 45–55 mmol/kg dry wt or equivalent to about 12 mM/l cell H_2O. The actin content of smooth muscle is about 1 mM and the ATP content of portal vein is less than 4 mM[9], hence, less than one-half of the total cytoplasmic Mg can be accounted for by the divalent cation bound to ATP and to F-actin[5]. Therefore, it would be surprising if *free* cytoplasmic [Mg^{2+}] in smooth muscle were significantly less than 1 mM, although the possibility of yet unidentified Mg buffers cannot be excluded.

Changes in cytoplasmic Mg have been observed with EPMA following prolonged (30 min) maximal contractions of PAMV and after exposure to Ca-free, high (16.2 mM) free Mg^{2+} solution[5]. In the contracted preparations, cytoplasmic Mg was slightly decreased, while in the high Mg^{2+}, Ca-free solution it was increased.

Acknowledgments. Supported by HL15835 and HL07499 (CV Training in Electron Optical Research) to the Pennsylvania Muscle Institute, a post-doctoral fellowship from the American Heart Association to J.W., and a postdoctoral fellowship from the Muscular Dystrophy Association to M.B.

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