Calcium-Sensitive Cellular and Subcellular Transport of Sodium, Potassium, Magnesium, and Calcium in Sodium-Loaded Vascular Smooth Muscle

Electron Probe Analysis

Arthur J. Wasserman, George McClellan, and Andrew P. Somlyo

SUMMARY Electron probe x-ray microanalysis of the composition of rabbit portal anterior mesenteric vein smooth muscle was performed following sodium loading and washout into sodium-free lithium solutions. Sodium and lithium were also measured with atomic absorption spectrophotometry. Cellular uptake of sodium and loss of potassium during sodium loading were much faster at high (37°C) than at low (2°C) temperature, as was the passive ouabain-resistant uptake of potassium during lithium washout. The loss of sodium at 2°C into lithium solution consisted of two components: a rapid efflux that was complete by 30 minutes, and a slow component that required at least 24 hours for completion. The amount of sodium lost through the first component (approximately 200–300 mmol/kg dry weight) was relatively independent of the amount of sodium loading. The loss of cellular sodium at 2°C, after 30 minutes, was accompanied by a gain of cellular lithium. Ouabain-resistant sodium loss and lithium and potassium uptake were markedly accelerated at 37°C; sodium loss was complete (1200 mmol sodium/kg dry weight lost) by 30 minutes of washout. Sodium-loaded cells also lost chloride ion and gained magnesium during sodium efflux at 37°C. Mitochondrial and nuclear sodium and potassium were correlated with the respective cytoplasmic concentrations during both sodium loading and sodium washout, indicating the relatively rapid equilibration of the monovalent ions between the cytoplasm and organelles. Calcium-free solutions markedly inhibited the ouabain-resistant sodium and chloride ion effluxes and potassium influx in muscles incubated, after sodium loading, in lithium solutions at 37°C. These fluxes could be restored to near normal values by 0.2 mM calcium. The calcium sensitivity of the ouabain-resistant sodium, potassium, and chloride ion fluxes observed in this and other studies raises the possibility that some abnormalities of monovalent ion transport observed in cells of hypertensives are secondary to changes in cellular calcium.


A SIGNIFICANT fraction of the cellular Na⁺ in smooth muscle is lost into Na⁺−free, Li⁺−containing solutions, even at low (2–4°C) temperatures that inhibit the Na⁺ pump (Junker et al., 1984). This relatively temperature-insensitive component of Na⁺ efflux into Li⁺ solution was recently demonstrated by electron probe microanalysis (EPMA)* in both normal rabbit portal anterior mesenteric vein (RPAMV) that has a relatively high normal cellular Na⁺ content, and in Na⁺−loaded guinea pig Taenia coli (Junker et al., 1984). Because abnormalities of Na⁺/Li⁺ countertransport in red blood cells have been observed in some subgroups of human hypertensives (Cusi et al., 1981; Canali et al., 1984; Cana

* Electron probe x-ray microanalysis measures total (bound and free) ions. As a matter of convenience, and in view of considerable evidence that monovalent ions are primarily in solution, we have adopted the conventional notation for intracellular ion concentrations (indicating the charged species) in the present manuscript, although it is clearly incorrect in the case of divalent cations nessa 1984), the characteristics of Na⁺/Li⁺ exchange in vascular smooth muscle are of some interest. Therefore, the present studies were designed to obtain further information about the mechanism of Na⁺,K⁺−ATPase-independent Na⁺ efflux into Li⁺ solutions in vascular smooth muscle. The major advantage of using EPMA is that cells are analyzed under direct vision, and interpretation is not limited by possible ambiguities due to cellular heterogeneity or inability of distinguishing cellular from extracellular ion fluxes. Furthermore, it is also possible to determine with EPMA the relationship between the mitochondrial, nuclear, and cytoplasmic Na⁺ and K⁺ concentrations, and to establish whether deviations from single compartment flux kinetics, measured with radioactive tracers (for review, Jones 1980), can be attributed to compartmentalization in organelles.

Our previous experiments lead to the hypothesis that a low affinity transport system mediates the rapid Na⁺ efflux into cold Na⁺−free, Li⁺ solutions...
Warm Na\(^{+}\) Loading and Warm Ouabain Li\(^{+}\) Washout with 0.2 mM Ca\(^{++}\) or 0 Ca\(^{++}\) and 2 mM EGTA

Tissues were Na\(^{+}\) loaded at 37°C in K\(^{+}\)-free solution containing 1 mM ouabain as described above. Experimental strips were transferred to Li\(^{+}\)-Krebs as described above, except Ca\(^{++}\) was reduced to 0.2 mM or omitted entirely, and 2 mM EGTA was added.

Cryoultramicrotomy and Electron Probe Microanalysis

Transverse cryosections were obtained with a (modified) LKB ultracrystome as previously described (Karp et al., 1982). Freeze-dried sections were mounted on carbon film covered 200-mesh copper grids, carbon coated, and analyzed on a Philips electron microscope (EM 400T) equipped with a Kevex Si(Li) x-ray detector and multichannel analyzer (Kevex Corp.), and interfaced to a PDP-11/34 computer. Analyses were performed on a Gatan liquid nitrogen-cooled cold holder maintained at -100°C, with careful attention to minimize microscope vacuum leaks (residual H\(_2\)O pressure 3.5 X 10\(^{-6}\) torr), ice depoosition on the cold specimen and mass loss due to etching (Shuman et al., 1976) are avoided under these conditions. Probe sizes were 1-1.5 \(\mu\)m for analysis of large areas of cytoplasm and nuclei, and 0.15 \(\mu\)m in diameter for the analysis of mitochondria.

The ratio of characteristic continuum x-rays generated from a specimen by the electron beam is a measure of the concentration of the element giving rise to the characteristic x-rays (Hall, 1971; Shuman et al., 1976, 1977). The method we use to quantify the x-rays is based on a computerized multiple least squares fit of an experimental spectrum to a reference file consisting of characteristic x-ray peaks generated from pure elements from which the background has been subtracted with a digital filter. To minimize the potential errors in quantification due to small drifts in detector calibration (whether a shift in centroid position on the energy axis or a change in detector resolution) and the overlap between the K\(^{+}\) K\(_a\) and the Ca\(^{++}\) K\(_a\) peaks, the first and second derivatives of the K\(^{+}\) x-ray peaks are included in the computer fit. This method permits accurate quantification of low concentrations of Ca\(^{++}\) in the presence of high concentrations of K\(^{+}\) (Kitazawa et al., 1983). In addition, we analyzed all carbon films used in this study to ascertain the absence of Ca\(^{++}\) contamination. These two factors allowed us to quantify cellular Ca\(^{++}\) in frozen thin sections with a precision of 0.3 mmol/kg dry weight. However, our analysis may slightly underestimate total cellular (as distinguished from cytoplasmic) Ca\(^{++}\), because probes were positioned at some distance from the plasma membrane to exclude extracellular contributions, and, consequently, did not include some of the junctional sarcoplasmic reticulum (SR) in the analyzed regions (Bond et al., 1984a, 1984b).

Total Na\(^{+}\) and Li\(^{+}\) contents of Na\(^{+}\)-loaded Li\(^{-}\)-washed tissues were also measured with an atomic absorption spectrophotometer (Varian, model AA575), because Li\(^{+}\) is not detected with conventional energy dispersive detectors with a Be window. Gently blotted tissues were weighed on preweighed aluminum foil and dried overnight at 110°C, and the dry weight was determined. Strips then were digested in 1 N nitric acid overnight, broken up by mortar and pestle, and spun down at 5000 g for 10 minutes. The supernatant was separated from the pellet and used for atomic absorption spectrophotometry. Cellular Na\(^{+}\) and Li\(^{+}\) were estimated as the difference between the total tissue electrolyte and the (measured) concentra-
tion in the respective solutions, based on an extracellular space of 43%, measured in electron micrographs of the entire cross-section of preparations used in this study. The difference between this value and the 50% 60Co-EDTA space (Jones et al., 1973) reflects the smaller extracellular space due to removal of the adventitia and of the intimal bands of connective tissue, in the present study. Na+ measurements were made after a 3-hour warm Na+ load and 16-hour cold or 30-minute warm Li+ washout; Li+ was measured after 30 minutes and 16 hours cold, or after 30 minutes warm (with 1 mM ouabain) Li+ wash.

Statistics

One- and two-way analysis of variance was used to partition the total experimental variation into components that could be identified with the experimental parameters and the variation attributable to experimental error. Since it was not feasible to maintain equal subclass numbers, we used the general procedure for analysis of variance with unequal subclass numbers (Harvey, 1960). To evaluate the effect of time on the changes in ion concentrations, simple or multiple linear regression procedures were used as applicable. The F statistic was used to test the presence of statistically significant differences between treatment groups and the significance of the regression coefficients. Differences were considered significant when the probability level, as determined by tables of F values, was less than 0.05 (P < 0.05). When statistically significant differences were shown to exist by analysis of variance, the significance of the difference was evaluated using Duncan's new multiple range test (Harvey, 1960).

There was a significant animal-to-animal variation in the measurements of the ion concentrations. In general, the K+, Na+, and Cl- concentrations differed significantly (P < 0.01) between animals. For example, the means of the [Na+] in RPAMV, Na+-loaded at 37°C, from eight animals ranged from 989 to 1328 with a mean of 1158 mmol/kg dry weight. One or more of the animals in this group had cytoplasmic [Na+] that differed from the others. This pattern was the same for K+ and Cl-, as well as for the other experimental groups. Possible sources of biological variations in the concentration of elements in solution have been discussed previously (Somlyo et al., 1979). There was very little variation in cellular Ca** between animals in any of the Na*-loaded groups. For the same warm Na*-loaded experimental group, the means of the [Ca**+] for the eight animals ranged from 0.96 to 2.12 mmol/kg dry weight, with no evidence of any statistically significant difference between them (P > 0.10): Animal-to-animal and cell-to-cell variations in cellular Ca** were large after 5-minute Na+ washout at 37°C in 1.2 mM Ca**-Li solution (see Results).

Results

Cold Na+ loading of RPAMV (and guinea pig Taenia coli) results in a morphologically and compositionally bimodal distribution of smooth muscle cells (Junker et al., 1984). Most cells resemble normal unloaded cells in density, and have a moderately high Na+, low K+, and normal Ca++ content. A variable, but small, number of 'light' (electronlucent) cells have very high Na+ and Ca++ contents, and very low concentrations of P. Because we had previously demonstrated that the 'light cells' represent a hyperpermeable and abnormal population (Junker et al., 1984), we have excluded all light cells from the data analyzed in this study. Most cryosections did not contain light cells, as illustrated in Figure 1, an electron micrograph of a typical cryosection of RPAMV used for analysis, showing smooth muscle cells, mitochondria and nuclei.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Electron micrograph of thin section of rabbit portal anterior mesenteric vein, cold (2°C Li+ washed for 24 hours. Seen are numerous transversely sectioned smooth muscle cells. Note mitochondria (arrow), nuclei (N), and collagen (asterisk).
### Table 1

<table>
<thead>
<tr>
<th>Composition of Na+-Loaded Cells at 2°C followed by Li+ Wash at 2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Na+ load, 2°C</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>No of animals</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>K+ mmol/kg dry wt</td>
</tr>
<tr>
<td>Na+ mmol/kg dry wt</td>
</tr>
<tr>
<td>Cl- mmol/kg dry wt</td>
</tr>
<tr>
<td>Mg++ mmol/kg dry wt</td>
</tr>
<tr>
<td>Ca++ mmol/kg dry wt</td>
</tr>
<tr>
<td>P mmol/kg dry wt</td>
</tr>
<tr>
<td>S mmol/kg dry wt</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. n = number of analyses.

### Composition of Cold Na+-Loaded and Cold Li+-Washed Rabbit Portal Vein

The results of electron probe analysis of RPAMV Na+ loaded at 2°C for 4 hours are shown in Table 1. Compared to the cytoplasmic Na+ (180 mmol/kg dry weight) in normal (non-Na+-loaded) cells (Junker et al., 1984), there was only a doubling of [Na+]i (uptake of 183 mmol/kg dry weight) during this period, with very large cell-to-cell variations in Na+ content. The frequency distribution of cytoplasmic Na+ (Fig. 2a) included a 15-fold range of Na+ concentrations, with a mean Na+ content of 363 mmol/kg dry weight. The (incomplete) Na+ loading appeared to be the result of Na+ exchange for K+, as also indicated by the highly significant (P < 0.001) negative correlation (r = -0.91) between cytoplasmic Na+ and K+. Even in the cells that had the highest [Na+]i, a significant amount of cytoplasmic K+ remained.

Standard multiple regression analysis was used to estimate the linear relationship between the Na+ and K+ concentrations in RPAMV loaded with Na+ at 2°C for 4 hours, loaded with Na+ at 37°C for 3 hours (see below), and normal (not Na+-loaded) tissue. The normal data from a previous series of EPMA-measured muscle (Bond et al., 1984b), kindly provided by Dr. Meredith Bond, were incorporated into the multiple regression analysis in order to extend the results from normal to Na+-loaded tis-

![Graph A](image-url)  
**A. In Cold Na+ Loaded Rabbit PAMV**

![Graph B](image-url)  
**B. In Warm Na+ Loaded Rabbit PAMV**
sues. To compensate for the known animal-to-animal variations in the Na\(^+\) and K\(^+\) concentrations, Cl\(^-\) concentration was included in the multiple regression analysis. There was no statistically significant change in [Cl\(^-\)] associated with either the Na\(^+\) loading or the Na\(^+\) washout. As a consequence, the variation in Cl\(^-\) concentration can be assumed to reflect the status of the cells independent of the concomitant changes taking place in Na\(^+\) and K\(^+\) concentrations. The multiple regression equation for the Na\(^+\)-loaded and the control muscles (from six animals) is:

\[ [K^+] = -(0.86 \pm 0.04)[Na^+] + (0.95 \pm 0.17)[Cl^-] + 607.9 \]

Both regression coefficients and the multiple correlation coefficient of 0.982 are highly significant (P < 0.001). This equation indicates that when the variation between animals is accounted for by holding the Cl\(^-\) concentration constant, there is essentially a one-for-one exchange of Na\(^+\) for K\(^+\) by the cells.

The total cell Ca\(^++\) (1.40 ± 0.2 mmol/kg dry weight) measured with large diameter (1–1.5 μm) probes was not significantly different in cold Na\(^+\)-loaded than in normal, resting RPAMV [1.3 ± 0.3 mmol/kg dry weight (Bond et al., 1984)]. The somewhat lower cell Na\(^+\), Cl\(^-\), and Ca\(^++\) contents of the cold Na\(^+\)-loaded cells in this than in our previous study (Table 7 in Junker et al., 1984) reflect the inclusion of analyses of light cells in the earlier work.

The composition of RPAMV, Na\(^+\) loaded at 2°C, at various intervals following washout in Li\(^+\)-Krebs solution at 2°C is shown in Table 1. Within 10 minutes of cold Li\(^+\) wash, cytoplasmic Na\(^+\) was reduced by 198 mmol/kg dry weight, paralleling our previous observations (Junker et al., 1984). The bulk of the Na\(^+\) gained during the loading period (54% of Na\(^+\)) was lost during 10 minutes, and [Na\(^+\)] did not change significantly thereafter for up to 2 hours in 2°C Li\(^+\) wash. The frequency distribution of cytoplasmic Na\(^+\) after cold, 30-minute Li\(^+\) wash varied over a 5-fold range (Fig. 3a). The sum of [Na\(^+\)]\(\text{b}\) and [K\(^+\)], was lower in the Li\(^+\)-washed than in the Na\(^+\)-loaded or non-Na\(^+\)-loaded normal (Junker et al., 1984) cells. Neither [K\(^+\)] nor [Cl\(^-\)] changed significantly during washout.

Composition of Warm Na\(^+\)-Loaded and Cold Li\(^+\) Washed Rabbit Portal Vein

When RPAMV was loaded with Na\(^+\) at 37°C, the [Na\(^+\)]\(\text{b}\) increased to 1158 mmol/kg dry weight (Table 2) and varied over a 3-fold range: this Na\(^+\) content is 3 times higher than in the cold Na\(^+\)-loaded cells and about 6 times higher than in the non-Na\(^+\)-loaded cells. Furthermore, the frequency distribution of Na\(^+\) concentration was normal in the cells that were Na\(^+\) loaded at 37°C (Fig. 2b), unlike the skewed distribution found after cold Na\(^+\) load (Fig. 2a). The exchange of K\(^+\) for Na\(^+\) was also much more complete at 37°C than at 2°C, and virtually all K\(^+\) had been lost from the cytoplasm (Table 2A) during the 3-hour Na\(^+\) load at 37°C. The [Cl\(^-\)], of 415 mmol/kg dry weight (Table 2) did not differ significantly (by analysis of variance) from the [Cl\(^-\)] in cold Na\(^+\)-loaded (321 mmol/kg dry weight; Table 1) or non-Na\(^+\)-loaded (399.6 mmol/kg dry weight) tissue (Junker et al., 1984). The cellular Ca\(^++\) (1.2 mmol/kg dry weight) was normal (Bond et al., 1984).

The Na\(^+\), K\(^+\), and Cl\(^-\) concentrations analyzed in 84 mitochondria from 6 animals and 26 nuclei from 5 animals all were closely correlated with the concentrations in the surrounding cytoplasm (Fig. 4). The mitochondrial and nuclear values were combined for the warm and the warm + ouabain, Na\(^+\)-loaded tissues, since there was no significant difference between these two groups. Mitochondrial Na\(^+\) (Fig. 4) was 779 ± 20.8 mmol/kg dry weight. The K\(^+\) and Cl\(^-\) were 4 ± 6 and 258 ± 11 mmol/kg dry weight, respectively. Nuclear Na\(^+\) was 1197 ± 47 and was as high as cytoplasmic Na\(^+\). Nuclear K\(^+\)
TABLE 2
Composition of Na+-Loaded Cells at 37°C followed by Li+ Wash at 2°C

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>n</th>
<th>Na+ load, 37°C</th>
<th>Li+ wash, 2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>mmol/kg dry wt</td>
<td></td>
<td>1158 ± 10</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>K+</td>
<td></td>
<td>37°C</td>
<td>Li+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44 ± 1</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>Mg++</td>
<td></td>
<td>209 ± 7</td>
<td>327 ± 12</td>
</tr>
<tr>
<td>Ca++</td>
<td></td>
<td>44 ± 7</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>265 ± 5</td>
<td>286 ± 6</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>277 ± 3</td>
<td>286 ± 6</td>
</tr>
</tbody>
</table>
| Results are expressed as mean ± SEM. n = number of analyses.

and Cl⁻ were 10 ± 2 and 349 ± 32 mmol/kg dry weight, respectively.

Cytoplasmic Na⁺ was reduced by 300 mmol/kg dry weight (26% of [Na⁺]), after a 10-minute cold Li⁺ wash, and did not change significantly during the next 110 minutes (Table 2). The frequency distribution of cytoplasmic Na⁺ in warm Na⁺-loaded and 30-minute cold Li⁺-washed tissues varied over a more than 3-fold range (Fig. 3b). However, after 18 hours, [Na⁺], was reduced by 949 mmol/kg dry weight and after 24 hours of cold Li⁺ wash, the tissues had lost a total of 1077 mmol Na⁺/kg dry weight, and [Na⁺] was well below normal values (Junker et al., 1984).

FIGURE 4. Mitochondrial (upper panels) [Na⁺] and [K⁺] in rabbit portal anterior mesenteric vein at 37°C, under various experimental conditions; Na⁺-loaded, normal (1.2 mM Ca⁺) Li⁺-washed + ouabain, O Ca⁺ + 2 mM EGTA + 1 mM ouabain Li⁺-washed and 0.2 mM Ca⁺ + 1 mM ouabain Li⁺-washed. The lower panels show the cytoplasmic [Na⁺] and [K⁺] in the same preparations.
During the first 2 hours in the cold Li⁺-washout solution, the cells gained K⁺ linearly at a rate of 0.3 mmol/kg dry weight/min. Although this linear uptake was statistically significant (r = 0.91), the amount of K⁺ moved was small relative to the total ion fluxes. The sum of the cytoplasmic Na⁺ and K⁺, after 24 hours in the Li⁺-washout solution, was 84% lower than the initial value in the warm Na⁺-loaded control (177 vs. 1104 mmol/kg dry weight). The chloride concentration remained essentially constant during the Li⁺-washout period. None of the Cl⁻ values differed significantly from each other or from those in the warm Na⁺-loaded tissues.

Nuclear and mitochondrial K⁺ also increased and was 105 ± 9 (n = 7) and 57 ± 4 (n = 16) mmol/kg dry weight by 24 hours, compared to the 10 ± 2 (n = 26) and 4 ± 6 (n = 84) mmol/kg weight initial, Na⁺-loaded values. Nuclear Na⁺ decreased to 77 ± 22 (n = 7) and mitochondrial to 43 ± 14 (n = 16) mmol/kg dry weight from the initial Na⁺-loaded values of 1197 ± 47 (n = 26) and 779 ± 21 (n = 84) mmol/kg dry weight, respectively, in parallel with the changes in cytoplasmic concentrations (Table 2).

Cytoplasmic (Table 2) and nuclear Ca²⁺ remained low, and by 24 hours, the latter was 0.8 ± 2 (n = 7) mmol/kg dry weight. These values never differed significantly from the values in either the Na⁺-loaded or non-Na⁺-loaded control tissues.

Four atypical cells in the 24-hour cold Li⁺ wash group were deleted from the tabulated values. In three of these cells, the cytoplasmic Ca²⁺ was abnormally high (16–29 mmol/kg dry weight) and a fourth cell had an abnormally low phosphorus content (81 mmol/kg dry weight). These Ca²⁺ and P values are typical of (hyperpermeable) "light cells," although these particular cells could not be so identified morphologically or through other aspects of composition (K⁺, Na⁺, Cl⁻).

Composition of Warm Na⁺-Loaded Rabbit Portal Vein in the Presence of 1 mM Ouabain

This experimental group was Na⁺ loaded at 37°C, in K⁺-free, 1 mM ouabain solution, to ensure that the Na⁺,K⁺ pump was fully inhibited during the subsequent Li⁺ wash at 37°C. The composition of these cells (Table 3) was not significantly different from those that were Na⁺ loaded at 37°C in the absence of ouabain (Table 2). Mitochondrial [Na⁺]⁺ and [K⁺] were 903 ± 26 and 3 ± 1 mmol/kg dry weight, respectively, and were not significantly different from the values in tissues Na⁺-loaded without ouabain. The combined values are illustrated in Figure 5.

The Ouabain-Insensitive Na⁺ efflux at 37°C from Cells Na⁺-Loaded at 37°C and Associated Ca²⁺ and Mg²⁺ Movements

Cytoplasmic Na⁺ was decreased by 938 mmol/kg dry weight after a 5-minute, ouabain-Li⁺ wash at 37°C (Table 3), and after 10 minutes, [Na⁺] had decreased by 1196 mmol/kg dry weight, or 94% of total [Na⁺] (Table 3). [Na⁺] continued to fall through the 30-minute period. The cytoplasmic Na⁺ concentrations of all Na⁺-loaded and Li⁺-washed tissues are presented graphically in Figure 5.

The changes in cytoplasmic [K⁺] were biphasic (Table 3). For the first 10 minutes, the [K⁺] rapidly increased and then decreased more slowly during the next 110 minutes. The precise shape of the curve describing the K⁺ fluxes cannot be defined, because of insufficient points. However, the simple linear regression of K⁺ vs. time through the 0-, 5-, and 10-minute points and the regression through the 10, 30, and 120 minutes were highly significant (P < 0.01).

Using the values from these regressions, the initial uptake of K⁺ was about 26 ± 6.0 mmol/kg dry weight per minute, and was followed by a decrease of −1.5 ± 0.3 mmol/kg dry weight per minute. The rate of K⁺ uptake and the total amount of K⁺ moved (c.f.: Tables 2 and 3), were much higher in the warm Li⁺-washout solution with ouabain than in the cold Li⁺-washout solution.

There was a significant (P < 0.001) decline in cytoplasmic Cl⁻ after a 5-minute washout (Table 3), but by 2 hours, Cl⁻ returned to the prewashout, Na⁺-loaded value.

At the earliest time sampled (5 minutes) during washout, mitochondrial and nuclear K⁺ were increased to 80 ± 6 (n = 80) and 307 ± 18 (n = 2).

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>mmol/kg dry wt</th>
<th>mmol/kg dry wt</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>K⁺</td>
<td>Na⁺</td>
</tr>
<tr>
<td><strong>Ouabain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺ load, 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>4 ± 1</td>
<td>1271 ± 30</td>
</tr>
<tr>
<td><strong>Li⁺ wash, 37°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>143 ± 9</td>
<td>333 ± 15</td>
</tr>
<tr>
<td>10 min</td>
<td>243 ± 13</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>30 min</td>
<td>201 ± 19</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>2 hr</td>
<td>116 ± 5</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± sem. n = number of analyses.
mmol/kg dry weight, respectively. Mitochondrial (Fig. 4) and nuclear Na⁺ and K⁺ changed in parallel with the respective cytoplasmic concentrations.

Cellular Ca²⁺ increased significantly ($P < 0.01$) to over 3 times the normal values, during the initial 5 minutes of Na⁺ efflux. There were very large variations among different cells and even within different areas of the same cell, probably reflecting the distribution of the sarcoplasmic reticulum. We emphasize that these high Ca²⁺ values include the contents of the sarcoplasmic reticulum, and should not be taken as representative of cytoplasmic Ca²⁺. After 5 minutes, the cellular Ca²⁺ declined and, by 2 hours, was not significantly different from the original, Na⁺-loaded values.

Cytoplasmic [Mg²⁺] increased significantly ($P < 0.001$) by 10 minutes of Li⁺ washout, and did not significantly change thereafter.
The Effect of Calcium on the Ouabain-Resistant Na⁺ Loss, on Cl⁻ and Ca++ Content, and K⁺ and Mg++ Gain in Warm Na⁺-Loaded Smooth Muscle

The loss of [Na⁺], and [Cl⁻], and gain of K⁺ were markedly reduced when washout was conducted in Ca++-free, EGTA-containing solutions (Table 4; Fig. 5). This reduction was apparent after both 5 and 10 minutes of washout. The addition of 0.2 mM Ca++ to the washout solution was sufficient to restore the movement of monovalent cations to nearly the same levels found in the presence of normal (1.2 mM) Ca++. Cellular Cl⁻ was significantly (P < 0.001) reduced during washout in 0.2 mM Ca++ (Table 4) or 1.2 mM Ca++-Li⁺ (Table 3), but not in Ca++-free solution, indicating a Ca++-sensitive Cl⁻ efflux.

Cellular Mg++ was significantly increased at 10 minutes when washout was conducted in Ca++-free or 0.2 mM Ca++ solutions. The increase in cytoplasmic Mg++ was greater in Ca++-free (EGTA) than in normal (1.2 mM Ca++) Li⁺ solution.

Cellular Ca++ was also significantly (P < 0.05) reduced in the Ca++-free, EGTA-containing solution. The increase in cell Ca++ in 0.2 mM Ca++ after 5-minute Na⁺ efflux was similar to that found in the presence of 1.2 mM Ca++ (c.f.: Tables 3 and 4).

Atomic Absorption Spectrophotometry of Warm Na⁺-Loaded and Cold or Warm Li⁺-Washed Rabbit Portal Vein

In tissues that were Na⁺ loaded at 37°C for 3 hours, cellular Na⁺ was 1210 mmol/kg dry weight, and was reduced to 113 mmol/kg dry weight after a 16-hour cold Li⁺ wash. Both of these values are in good agreement with the results of electron probe analysis (Table 2). Cellular Li⁺ was 191 and 916 mmol/kg dry weight after a 30-minute and a 16-hour cold Li⁺ wash, respectively. This is comparable to the amount of cytoplasmic Na⁺ lost, and the summed [Na⁺] + [Li⁺] + [K⁺], at 16 hours (1194 mmol/kg dry weight, using [K⁺] values at 18 hours from Table 2) is similar to the value of [Na⁺] + [K⁺] in the Na⁺-loaded muscles.

After a 30-minute 37°C Li⁺ wash containing 1 mM ouabain, Na⁺ was reduced to 55 mmol/kg dry weight, [Li⁺], was 670 mmol/kg dry weight, and [Na⁺] + [K⁺] + [Li⁺] was 926 mmol/kg dry weight. This value is lower than [Na⁺] + [K⁺] in the Na⁺-loaded muscles, and is consistent with the transient reduction in cell Cl⁻ (Table 3) during the early phase of Na⁺ washout.

Discussion

Temperature Dependence of Cytoplasmic Na⁺ Movements and Evidence of Li⁺ Exchange for Na⁺

Both the passive influx of Na⁺ into smooth muscle and a large component of the (ouabain-resistant) Na⁺ efflux were sensitive to temperature: Na⁺ loading in K⁺-free solutions and Na⁺ efflux into Li⁺ solutions were markedly reduced at 2°C, whereas, at 37°C, the exchange of [Na⁺] for K⁺ was essentially complete within 2 hours (Table 2). The cellular Na⁺ concentrations after, respectively, cold (4 hours) and warm (3 hours) Na⁺ loading were (in mmol/kg dry weight) 363 and 1158. The pronounced temperature sensitivity of the Na⁺ channels through which passive influx occurs is frequently unrecognized, although some electrophysiological and ion flux studies (Kao and Nishiyama, 1969; Daniel and Robinson, 1971) have shown the temperature sensitivity of K⁺ and Na⁺ permeability in uterine smooth muscle. Other ouabain-resistant transport systems, for example, Li⁺ efflux in human red blood cells (Levy and Liune, 1984), show similarly high temperature sensitivity.

Moderately Na⁺-loaded (in the cold) smooth muscles lost approximately 200 mmol Na⁺/kg dry weight during efflux into Li⁺ solution at low temperature. This cellular Na⁺ loss was apparently complete within 10 minutes, and showed little or no further change for up to 2 hours. Somewhat surprisingly, the amount of cellular Na⁺ lost (approximately 300 mmol Na⁺/kg dry weight) from massively Na⁺-loaded (at 37°C) cells was not significantly different, and the detectable early Na⁺ loss also occurred within the first 10 minutes (Table 2). Thus, the nearly four times higher intracellular Na⁺ content of warm Na⁺-loaded cells did not result in the nearly 4-fold increase in the rate of Na⁺ efflux, predicted.

<table>
<thead>
<tr>
<th>Table 4</th>
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<tbody>
<tr>
<td><strong>Effects of Ca²⁺-Free or 0.2 mM Ca²⁺ Solution on Cell Composition during Li⁺ Washout at 37°C (with 1 mM Ouabain)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of</th>
<th>mmol/kg dry wt</th>
<th>K⁺</th>
<th>Na⁺</th>
<th>Cl⁻</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>P</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ouabain</strong></td>
<td>2</td>
<td>37</td>
<td><strong>4 ± 1</strong></td>
<td>1271 ± 30</td>
<td>496 ± 19</td>
<td>48 ± 2</td>
<td>1.6 ± 0.3</td>
<td>294 ± 6</td>
</tr>
<tr>
<td>Na⁺-loaded, 37°C</td>
<td>2</td>
<td>37</td>
<td>28 ± 0</td>
<td>805 ± 24</td>
<td>534 ± 17</td>
<td>64 ± 3</td>
<td>0.4 ± 0.4</td>
<td>266 ± 8</td>
</tr>
<tr>
<td>Li⁺ wash, 37°C</td>
<td>5 min, EGTA</td>
<td>2</td>
<td>35</td>
<td>87 ± 5</td>
<td>254 ± 15</td>
<td>372 ± 25</td>
<td>37 ± 3</td>
<td>7.0 ± 1.1</td>
</tr>
<tr>
<td>5 min, 0.2 mM Ca²⁺</td>
<td>2</td>
<td>37</td>
<td>44 ± 2</td>
<td>694 ± 19</td>
<td>530 ± 26</td>
<td>74 ± 2</td>
<td>0.7 ± 0.4</td>
<td>323 ± 7</td>
</tr>
<tr>
<td>10 min, EGTA</td>
<td>2</td>
<td>42</td>
<td>151 ± 9</td>
<td>132 ± 13</td>
<td>228 ± 14</td>
<td>78 ± 3</td>
<td>2.8 ± 1.0</td>
<td>279 ± 13</td>
</tr>
<tr>
<td>10 min, 0.2 mM Ca²⁺</td>
<td>2</td>
<td>35</td>
<td>228</td>
<td>530 ± 26</td>
<td>74 ± 2</td>
<td>0.7 ± 0.4</td>
<td>323 ± 7</td>
<td>298 ± 5</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE, n = number of analyses.
by a simple, first-order model. The massive Na\(^+\) loss originates from a structurally identifiable compartment, such as mitochondria or nuclei, since the Na\(^+\) content of organelles was well correlated with the cytoplasmic concentration.

A major portion of Na\(^+\) efflux occurred in exchange with Li\(^+\), as indicated by the increase in cell Li\(^+\) accompanying Na\(^+\) loss. The exchange of Li\(^+\) for Na\(^+\) has also been observed, albeit at slower rates, in rat tail artery smooth muscle (Friedman, 1977). In *Taenia coli*, next to Na\(^+\), Li\(^+\) is the most effective counter-ion for Na\(^+\) exchange (Brading, 1975). It is unlikely that Na\(^+\) efflux was coupled to bicarbonate movement, because more recent studies also show Ca\(^++\)-sensitive Na\(^+\) efflux in bicarbonate-free solutions (Kennedy et al., 1986). However, a fraction of Na\(^+\) efflux at 37°C may also have occurred with Cl\(^-\) as the co-ion, in view of the transient Cl\(^-\) loss (Tables 3 and 4). *Taenia coli*, a phasic and spontaneously active smooth muscle similar to portal vein, is depolarized after a 3-hour Na\(^+\)-loading period (Casteels et al., 1973) comparable to that used in our study. Transfer of depolarized portal vein smooth muscle into the Li\(^+\) solution (present study) could result in hyperpolarization, if P_{Li} were lower than P_{Na}, although the presence of K\(^+\) in the Li\(^+\) solution would tend to oppose this through its depolarizing effect. Hyperpolarization could cause the transient decrease in cell Cl\(^-\) during Li\(^+\) washout (Table 3).

Only a very small fraction of the Na\(^+\) lost during Li\(^+\) washout from RPAMV could be compensated for by the (modest) influx of K\(^+\) and Mg\(^2+\). Only about 100 mmol K\(^+\)/kg dry weight were gained at a time when the cells have lost 1100–1200 mmol Na\(^+\)/kg dry weight. The fact that the amount of passive K\(^+\) gain that occurred in 5 minutes at 37°C (in the presence of ouabain) was equivalent to that which occurred during 18 hours at 2°C is probably also indicative of the temperature sensitivity of passive membrane K\(^+\) permeability. The uptake of Mg\(^2+\) was greatest during Li\(^+\) washout with Ca\(^++\)-free solutions at high temperatures (Table 3), and accounted for, at most, 50 mEq Na\(^+\)/kg dry weight. An Na\(^+\)/Mg\(^2+\) exchange system transporting Mg\(^2+\) in the outward direction and energized by an inward Na\(^+\) gradient is thought to be present in vascular smooth muscle (Palayt, 1974). Our results suggest that Na\(^+\)/Mg\(^2+\) exchange can also occur in the reverse direction, although some of the Mg\(^2+\) influx, in Ca\(^++\)-free solutions, is probably passive (Palayt, 1974; Bond et al., 1984b)

### Ca\(^++\) Sensitivity of Na\(^+\), Cl\(^-\), and K\(^+\) Movements

The Ca\(^++\) dependence of ouabain-resistant Na\(^+\) efflux was revealed unexpectedly in a study of mitochondrial diveralant cation movements (A.P. Somlyo, A.J. Wasserman, T. Fujimori, R. Broderick, in preparation), where we found that Na\(^+\) loss from massively Na\(^+\)-loaded cells was markedly inhibited in Ca\(^++\)-free (EGTA-containing) solutions (Table 4; Figs. 4 and 5). After an initial loss of approximately...
470 mmol Na⁺/kg dry weight during the first 5 minutes, no further significant Na⁺ loss was observed at 10 minutes, during washout into the Ca²⁺-free solution. In contrast, during the same 10-minute interval, nearly all (about 1200 mmol Na⁺/kg dry weight) of the Na⁺ was lost in normal (1.2 mm) Ca²⁺ solutions. The ouabain-resistant Na⁺ loss was restored to nearly normal values by 0.2 mm Ca²⁺ (Fig. 5). A large reduction (from 10⁻³ to 10⁻⁸ M) in extracellular Ca²⁺ will also reduce cytoplasmic free Ca²⁺ (Lopez et al., 1983; Kobayashi et al., 1985), and it is likely that the inhibition of Na⁺ efflux by Ca²⁺-free (EGTA) solutions was due to secondary reductions in cytoplasmic free Ca²⁺. Both the Na⁺/Li⁺ countertransport in red blood cells (Canessa, 1984) and Na⁺-H⁺ exchange (Mahnensmith and Aaronson, 1985) are modulated by cytoplasmic Ca²⁺. It remains to be determined to what extent the Ca²⁺-sensitive, ouabain-resistant Na⁺ efflux in smooth muscle (present study) is mediated by these pathways.

The inhibition of passive K⁺ influx by Ca²⁺-free (EGTA) solutions (Table 4) was, presumably, also due to the inhibition of Ca²⁺-activated K⁺ channels. The Cl⁻ efflux was also highly Ca²⁺ sensitive and, unlike the partially inhibited Na⁺ efflux, it was completely blocked in Ca²⁺-free solution (Table 4). A Ca²⁺-dependent Cl⁻ flux has also been observed in rat aorta (Smith and Jones, 1985). It is now clear that, as in other systems, cytoplasmic free Ca²⁺ modifies, directly or indirectly, the permeability of the plasma membrane to several ions (Hauerslev, 1983; Bolton and Clapp, 1984; Aaronson and Jones, 1985; Casteels and Droogmans, 1985; Smith and Jones, 1985; present study). Consequently, the question arises whether alterations of monovalent cation transport (e.g., Na⁺/Li⁺ countertransport) in various disease states, including hypertension, are primary manifestations of the disease or secondary to increases in [Ca²⁺].

Mitochondrial and Nuclear Na⁺ and K⁺ in Na⁺-Loaded Smooth Muscle and during Na⁺ Efflux

The mitochondrial and cytoplasmic concentrations of the major monovalent cations in normal smooth muscle are similar; the slightly lower mitochondrial K⁺ and Na⁺ (on a dry weight basis) is accounted for largely by the lower water content of mitochondria (Somlyo et al., 1979). In Na⁺-loaded preparations, mitochondrial K⁺ was lost and Na⁺ increased in parallel with the changes found in the cytoplasm. The time courses of the loss of mitochondrial and cytoplasmic Na⁺ were also indistinguishable during washout into warm Li⁺ solution. Similarly, the small and rapid (within 5 and 10 minutes) increase in cytoplasmic K⁺ that was due to ouabain-resistant fluxes was accompanied by a proportional change in mitochondrial K⁺ content. These findings, like those of earlier EPMA studies showing mitochondrial uptake of K⁺ and Rb⁺ in intact smooth muscle (Somlyo et al., 1979; Bond et al., 1984b; Kowarski et al., 1985), indicate the presence of relatively rapid mitochondrial exchange mechanisms for K⁺ and Na⁺ in situ. This behavior is markedly different from that of isolated liver mitochondria that show extremely low levels of (passive) K⁺ exchange (for review, Scarpa, 1979), but are consistent with the operation of active mitochondrial K⁺ transport systems (Jung et al., 1977; Garlid, 1980; Beavis et al., 1985). Nuclear concentrations of K⁺ and Na⁺ in normal smooth muscle are similar to the cytoplasmic concentrations, with minor differences possibly due to variations in local hydration and some (limited) cation binding by the nuclear matrix (Somlyo et al., 1979). As the latter communicates through relatively large nuclear pores with the cytoplasm, it was not surprising that the loss of nuclear Na⁺, from massively Na⁺-loaded cells, also paralleled the decrease in cytoplasmic Na⁺. The close correlation between the Na⁺ and K⁺ transients in, respectively, the cytoplasm and the cell organelles, together with the absence of significant Na⁺ compartmentalization in the SR in smooth muscle (Kowarski et al., 1985), indicates that anomalies of isotopic K⁺ and Na⁺ or Cl⁻ fluxes that depart from single compartment behavior are not due to compartmentalization in ultrastructurally identifiable organelles.

Cellular Ca²⁺ in Na⁺-Loaded Smooth Muscle and during Na⁺ Efflux into Ca²⁺-Containing and Ca²⁺-free Solutions

Cellular Ca²⁺ (1.2–1.4 mmol/kg dry weight) was not significantly different from normal values [1.3 mmol/kg dry weight (Bond et al., 1984b)], whether the Na⁺ loading was performed at low (see also Junker et al., 1984) or high (37°C) temperature. We reemphasize that the inclusion of the (increased) Ca²⁺ content of hyperpermeable (light) cells would be unavoidable in bulk (atomic absorption spectrophotometric or ⁴⁰Ca⁺ flux) measurement, and could lead to the erroneous interpretation that cell Ca²⁺ is increased in Na⁺-loaded smooth muscle. The absence of Ca²⁺ uptake during massive Na⁺ loading or with more modest degrees of Na⁺ efflux into Li⁺ solution argues, like observations on _Taenia coli_ (Raeymaekers et al., 1974; Aaronson and van Bree- man, 1981), against Na⁺-Ca²⁺ exchange playing a major, physiological role in RAPMV smooth muscle. The reduction in cell Ca²⁺ within 5 minutes in Ca²⁺-free solutions, in the presence of massive Na⁺ efflux, also indicates the existence of Ca²⁺ efflux pathways that are more active than Na⁺-Ca²⁺-exchange.

The substantial (approximately 3-fold) increase in cellular Ca²⁺ (at 37°C) during the first 5 minutes of Na⁺ efflux into Ca²⁺-containing Li⁺ solutions is consistent with results obtained with other methods showing Ca²⁺ influx during complete Na⁺ withdrawal (Aaronson and van Bree- men, 1981), and
with earlier studies (Somlyo and Somlyo, 1971) in which Na\(^+\) withdrawal was used to induce divalent cation (strontium) loading of the sarcoplasmic reticulum in smooth muscle. The subsequent loss of the Ca\(^{2+}\) gained (Table 3) is further indication of the ability of vascular smooth muscle to actively extrude Ca\(^{2+}\), even in the absence of extracellular Na\(^+\).

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INDEX TERMS: Mitochondria • Nucleus • Lithium • Ion transport
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