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SODIUM METABOLISM IN RAT RESISTANCE VESSELS

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SUMMARY

1. Using ^{22}Na we have investigated the Na metabolism in rat mesenteric resistance vessels (internal diameter about 200 μm).

2. The intracellular Na content was determined by washing the vessels at 0 °C in saline with Li substituted for Na, and was related to smooth muscle volume determined from measurements of media thickness and internal diameter using light microscopy. On this basis an internal Na concentration of 13·7 mmol l^{-1} cells was found.

3. The efflux of Na into saline at 37 °C consists of two phases, a fast one and a slow one, where the slow phase was considered to be intracellular. It had a rate constant of 0·137 min^{-1}, which was decreased by 50 % if 1 mM-ouabain was added to or K withdrawn from the efflux medium.

4. The gain of cell Na after addition of 1 mM-ouabain was much faster than would be expected from the decrease in efflux, indicating that 1 mM-ouabain increases the permeability to Na.

INTRODUCTION

Recent findings have suggested that hypertension may be associated with abnormal Na metabolism (Jones, 1973; Friedman & Friedman, 1976; Garay, Dagher, Pernollet, Devynck & Meyer, 1980; Canessa, Adragna, Solomon, Connolly & Tosteson, 1980). Until now, however, all information about vascular Na metabolism has been obtained from relatively large blood vessels such as aorta (Jones, 1973), the rabbit ear artery (Droogmans, Raeymaekers & Casteels, 1977) and rat tail artery (Friedman, 1974), vessels which are too large to play a role in the determination of peripheral resistance. It is therefore of importance that the Na metabolism of smaller vessels be examined. The purpose of the present study has been to investigate, in normotensive rats, Na metabolism (intracellular Na concentration, $[Na]_i$, as well as active and passive plasmalemma Na fluxes) in vessels with internal diameter about 200 μm and therefore small enough to contribute significantly to the determination of peripheral resistance (Folkow, Hallbäck, Jones & Sutter, 1977). Active and passive fluxes were distinguished on the basis of their sensitivity to 1 mM-ouabain, the high concentration being necessary owing to the insensitivity of rat Na–K-ATPase to this glycoside (Detweiler, 1967).

METHODS

Preparation

Segments (*ca.* 2 mm long) of mesenteric resistance vessels with internal diameter about 200 μm (2nd and 3rd generation vessels of the superior mesenteric artery) were taken from 10- to 15-week-old male Wistar rats. Unless otherwise stated the vessel segments were mounted in a myograph (Mulvany & Halpern, 1976) by threading them on two stainless-steel wires (diameter 40 μm) which were attached to a force transducer and a micrometer, respectively, and placed in an organ bath. The vessels were then stretched in physiological saline (see below) to a wall tension of *ca.* 0·2 mN mm^{-1}, where wall tension is force per wall length. In most cases, after determination of the vessel's morphological characteristics (see below), the vessel segments were de-mounted by removing one of the wires, keeping the other as a handle for transferring the vessel segments through the different solutions. In the other cases, the vessel internal circumference was set to a value (normalized internal circumference) where active response was maximal and the mechanical properties determined as described previously (Mulvany & Halpern, 1977).

Solutions

The *physiological saline* used in this study had the following composition (in mM): NaCl, 119; KCl, 4·7; $NaHCO_3$, 25; KH_2PO_4, 1·18; $MgSO_4$, 1·17; $CaCl_2$, 1·6; ethylenediaminetetraacetic acid (EDTA), 0·026; glucose, 5·5. *K saline* was physiological saline but with KCl substituted for NaCl on an equimolar basis and containing 2·5 mM-$CaCl_2$. *K-free saline* was physiological saline but with NaCl and NaH_2PO_4 substituted for KCl and KH_2PO_4 on an equimolar basis. *Li saline* was physiological saline but with NaCl and $NaHCO_3$ replaced by 107 mM-LiCl and 25 mM-Li_2CO_3. Solution used for loading ([^{22}*Na*]*saline*) was physiological saline containing ^{22}Na (Radiochemical Centre, Amersham) with a specific activity of 0·63 Ci mol^{-1}. All solutions except [^{22}Na]saline were bubbled with 5% CO_2 in O_2 to give pH 7·4. The vials containing [^{22}Na]saline were placed in a container held at 37 °C through which 5% CO_2 in O_2 saturated with water was circulated. Where indicated, ouabain (1 mM, Merck) was added to the solutions.

Estimate of smooth muscle cell volume

With the vessels mounted in the myograph it was possible using light microscopy to visualize the vessel wall and the media–adventitia border in the wall (Pl. 1). It was therefore possible to determine the effective internal circumference (L) (and from L the effective internal diameter (l) as $l = L/\pi$), the wall thickness (w) and the media thickness (m). The segment volume (V_s) and media volume (V_m) were then determined using the following formulae:

$$V_s = a(\pi w^2 + Lw)$$
$$V_m = a(\pi m^2 + Lm)$$

where a is the segment length.

Muscle cell volume (V_c) was estimated as 0·71 V_m, on the basis that 71% of the media consists of smooth muscle cells (Mulvany, Hansen & Aalkjær, 1978).

Determination of $[Na]_i$

After determination of the morphological characteristics the vessel segments were de-mounted and placed in physiological saline at 37 °C for at least 1 h. They were then placed in 40 μl [^{22}Na]saline for the times indicated. After this micro-incubation they were washed through a series of vials with Li saline at 0 °C and finally placed in a vial containing distilled water (Fig. 1*A*). The vials were filled with 5 ml Unisolve E scintillation liquid (Koch-Light Lab.), counted in a liquid scintillation counter (Mark III, model 6880, Searle Analytic Inc.) and the results corrected for quenching by external standardization.

The washout had two phases (Fig. 1*B*), an initial fast one probably representing washout of extravasal and extracellular ^{22}Na and a second slow one (rate constant = 0·0049 ± 0·0005 (S.E. of mean) min^{-1} (forty-four vessels)) representing washout from the intracellular space (Friedman, 1974). By back-extrapolation to time 0 of the slow phase a ^{22}Na pool was determined which, based on the findings of Friedman (1974), was considered as intracellular. The $[^{22}Na]_i$ was then determined from

$$[^{22}Na]_i = \frac{c}{sV_c}.$$

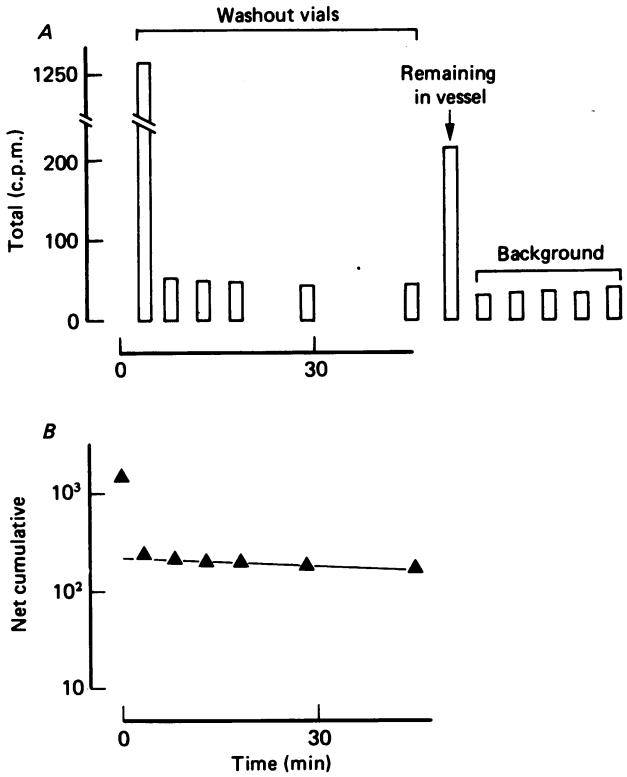

Fig. 1. *A*, principle of the wash procedure. After loading with ^{22}Na the vessels had three short rinses (1–2 s) in Li saline at 0 °C (not shown). They were then washed in Li saline (1·5 ml per vial) at 0 °C for 45 min (Friedman, 1974). The bars show the counts left in the washout vials, and those remaining in the vessel in a typical experiment. The last five bars represent the background measured in five vials containing 1·5 ml Li saline. *B*, washout curve calculated from the data in *A*. After subtraction of the background from the counts in the vials, a washout curve was constructed by sequentially adding the net counts in the washout vials in reverse order to the net counts from the vessel. A line was then fitted to the last four points and the intercept of this line with the ordinate was taken as the intracellular c.p.m.

In control experiments an attempt to slow the Na loss from the cells even further was made by adding ouabain to the Li saline, but this caused no measurable decrease in the efflux rate.

$[Na]_i$ was taken as the $[^{22}Na]_i$ after [^{22}Na]saline incubation for 30 min or more, at which time (see below) the intracellular and extracellular specific activity was similar.

In some experiments where indicated, attempts were made to release all intracellular Na. This was done by disrupting the cell membrane either (*a*) by alternate freezing (2 s in liquid nitrogen) and thawing (in Li saline at 1 °C) six times (Friedman, 1974) or (*b*) by incubating the vessels in distilled water at 37 °C for 3 h.

Determination of Na efflux

The vessels were first incubated as already described in [^{22}Na]saline. The vessels were then given two short rinses (30 s) in physiological saline at 37 °C, after which they were washed through a series of vials with physiological saline (1·5 ml per vial) at 37 °C for 15 min, and finally placed in a vial with distilled water. From the activity in the vials, washout curves were constructed as described in Fig. 1 and expressed as $[^{22}Na]_i$. The efflux rate was then determined as the slope of a line fitted to the washout curve between 4 and 15 min.

In another series of efflux experiments, where ouabain was added to the washout medium after 7 min washout in physiological saline, the washout curves were expressed as the instantaneous rate constant (Brading, 1972). Average rate constants were calculated as the geometric means of the instantaneous rate constants in the last 3 min before (*total rate constant*) and the first 3 min after addition of ouabain (*ouabain-resistant rate constant*) and the *ouabain-sensitive rate constant* was then calculated as the difference. In some experiments, instead of adding 1 mM-ouabain the efflux medium was changed to K-free saline after 7 min and the *K-sensitive rate constant* calculated as described for the ouabain-sensitive rate constant.

Statistics

The distribution of $[Na]_i$ and the rate constants were tested for normality before and after logarithmic transformation using a χ-square test (Sokal & Rohlf, 1969). This showed that $[Na]_i$ and the rate constants were geometrically distributed and the means were therefore calculated as geometric means. In Results, values are given as mean $\pm$ S.E. of mean or as geometric mean with the limits of S.E. of mean indicated in square brackets; the number of vessels is shown in parentheses. Differences between observed means were tested with the 2-tailed t test. The regression lines used for determination of zero intercepts were determined using least-squares regression.

RESULTS

Mechanical response

The normalized internal diameter of fourteen representative vessels was 192 ± 11 μm and the active wall tension response to K saline was $2{\cdot}18 \pm 0{\cdot}19$ mN mm^{-1}. Using Laplace's law ($p = T/r$ where p is pressure, T is wall tension and r is radius) this is equivalent to an active pressure of $22{\cdot}9 \pm 1{\cdot}4$ mN mm^{-2}, which is well in excess of the physiological demand. Furthermore, in a few experiments we de-mounted the vessels after determination of the active mechanical response, incubated them in the usual way in 40 μl physiological saline for 2 h and then mounted them on the myograph again. A new K saline response elicited at this time was not decreased, compared to the first, indicating that the vessels were still in good condition after micro-incubation.

Vessel volume and dry weight

In eleven experiments, after determination of their morphological characteristics, vessels were de-mounted, blotted between two pieces of filter paper and placed on a balance (Kahn electrobalance, model RG). Within 5 min the weight fell to a value which changed by less than 10% when held overnight at 100 °C (Fig. 2). We took this value ($13{\cdot}3 \pm 1{\cdot}3$ μg) as the dry weight of the vessels. The dry weight/vessel volume ratio and the dry weight/smooth muscle cell volume ratio is shown in Table 1.

Na uptake and $[Na]_i$

The time taken for the ^{22}Na to equilibrate with the intracellular Na is shown in Fig. 3. From this curve a unidirectional Na uptake was calculated as the mean uptake per minute over the first 2 min (Table 1) before back diffusion of ^{22}Na played a major role. In forty-four experiments the $[Na]_i$ was calculated from the ^{22}Na content after a 30 min equilibration period. The distribution of these values is shown in Fig. 4. A logarithmic transformation showed the best fit to a normal distribution, and the average was therefore calculated (see Methods) as the geometric mean (Table 1). To

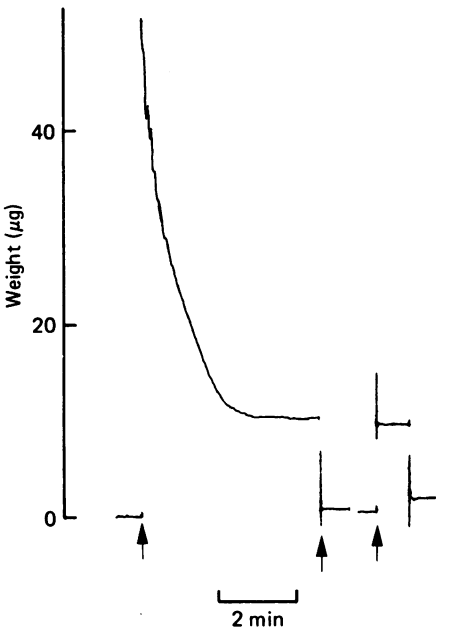

Fig. 2. Recording of a typical dry weight determination. Immediately after blotting between two pieces of filter paper the vessel was placed on the balance at room temperature (indicated by the first arrow). Due to evaporation the weight fell initially very rapidly, and reached after *ca.* 3 min a constant value which was taken as the dry weight. At the second arrow the vessel was removed from the balance and held overnight at 100 °C. At the third arrow the vessel was weighed again at room temperature.

TABLE 1. Na metabolism and morphological parameters of mesenteric resistance vessels. $[Na]_i$ and the Na fluxes in rat mesenteric resistance vessels are given as geometric means with the intervals in square brackets indicating the limits of S.E. of mean. The dry weight/vessel volume and the dry weight/smooth muscle cell volume ratios are given as means ± S.E. of mean. The numbers of observations are given in parentheses

$[Na]_i$	mmol l^{-1} cells	13·73 [12·96–14·54]	(44)
Unidirectional influx	mmol l^{-1} cells min^{-1}	2·41 [2·05–2·82]	(10)
Unidirectional efflux	mmol l^{-1} cells min^{-1}	1·95 [1·64–2·31]	(9)
Total rate constant	min^{-1}	0·137 [0·124–0·151]	(8)
Ouabain-resistant rate constant	min^{-1}	0·058 [0·051–0·065]	(8)
Ouabain-sensitive rate constant	min^{-1}	0·060 [0·042–0·086]	(8)
K-sensitive rate constant	min^{-1}	0·065 [0·060–0·070]	(28)
Dry weight/vessel volume	kg l^{-1}	0·334 ± 0·012	(11)
Dry weight/smooth muscle cell volume	kg l^{-1}	0·660 ± 0·041	(11)

evaluate to what extent the vessel size influenced the results, the smooth muscle volumes were grouped in three groups according to size ($V_m < 8{\cdot}5 \cdot 10^{-3}$ mm^3 ($n = 13$), $8{\cdot}5 \cdot 10^{-3}$ mm^3 $< V_m < 12 \cdot 10^{-3}$ mm^3 ($n = 20$) and $V_m < 12 \cdot 10^{-3}$ mm^3 ($n = 11$)). The $[Na]_i$ of these three groups was 14·2 [12·9–15·6], 14·3 [12·8–16·0] and 12·3 [10·8–13·0] mmol l^{-1} cells, respectively, and not significantly different. In control experiments the cell membrane was disrupted either by alternate freezing and thawing or by

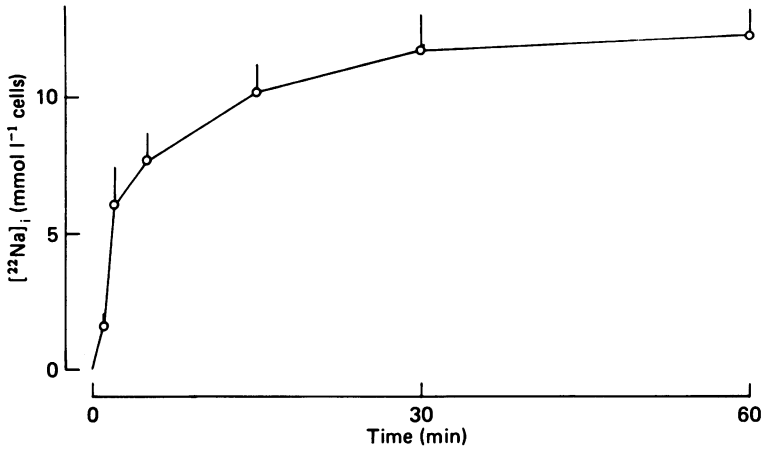

Fig. 3. Time course of net uptake of ^{22}Na. The values are determined using the cold Li method. Each point shows the mean of ten vessels, bars indicate S.E. of mean. From the mean uptake in the first and second minute the unidirectional influx (mmol l^{-1} cells min^{-1}) was estimated.

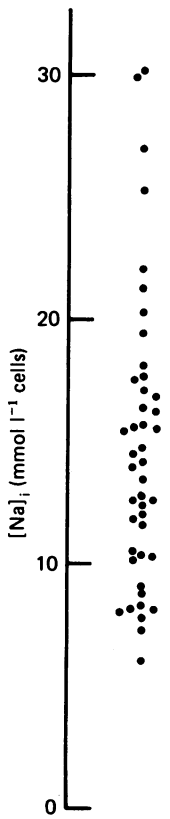

Fig. 4. Point diagram of the distribution of $[Na]_i$ in forty-four vessels after equilibration in [^{22}Na]saline for 30 min.

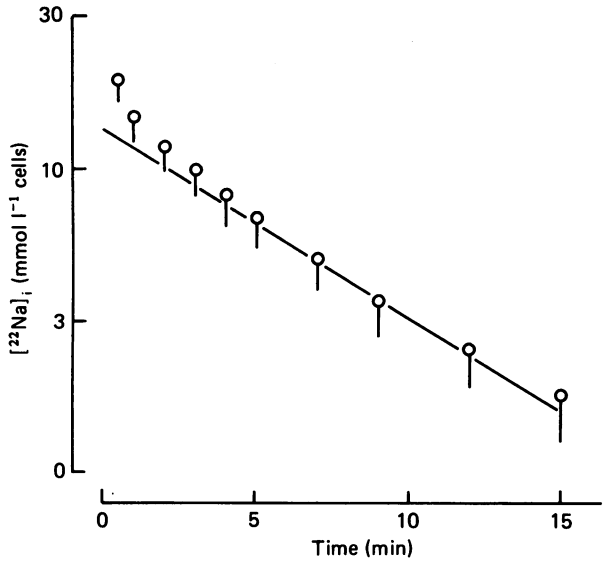

Fig. 5. Efflux of ^{22}Na into physiological saline at 37 °C. Each point shows the mean of eleven experiments, vertical bars indicate S.E. of mean. The line is calculated for values between 4 and 15 min inclusive.

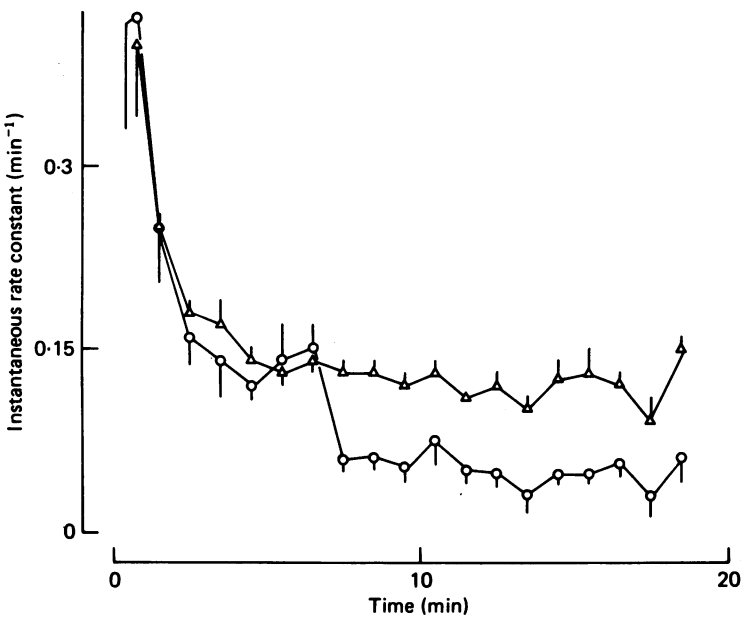

Fig. 6. Efflux of ^{22}Na into physiological saline (△) or into physiological saline to which 1 mM-ouabain was added after the initial 7 min wash period (○), expressed as the instantaneous rate constant. The points are the mean of eight experiments. Bars show S.E. of mean where they exceed size of symbols.

incubation in distilled water (see Methods). The $[Na]_i$ measured in these vessels was 1·3 [1·1–1·5] (10) and 1·8 [1·5–2·3] (6) mmol l^{-1} cells, respectively.

Na efflux

In a new series of experiments, vessels were equilibrated with ^{22}Na for 30 min and then washed in physiological saline at 37 °C (Fig. 5). The efflux curve could be split up in two phases. Phase 1 was very rapid and probably represented washout of the extravasal and extracellular ^{22}Na. Phase 2 was therefore considered to represent

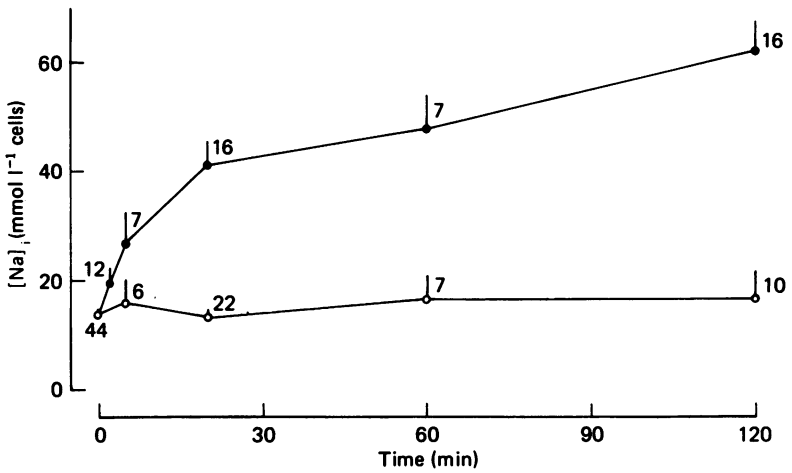

Fig. 7. Effect of 1 mM-ouabain on $[Na]_i$. The open circles are control determinations of $[Na]_i$. At time 0 the vessels had been equilibrated in [^{22}Na]saline for 30 min. The filled circles are the $[Na]_i$ in the presence of ouabain added at time 0. Vertical bars indicate S.E. of mean and the figures the number of vessels.

washout from the intracellular Na pool, and the $[Na]_i$ calculated from the back extrapolated intercept with the ordinate was 13·5 [11·0–16·6] mmol l^{-1} cells (11) and not significantly different from the value found using the cold Li method. From these experiments the unidirectional efflux was calculated (Table 1).

Fig. 6 shows the effect of ouabain on the unidirectional Na efflux. An immediate drop in the instantaneous rate constant in the first minute after exposure to ouabain was found, and the instantaneous rate constant was thereafter maintained. From these experiments the total rate constant and the ouabain-resistant and -sensitive rate constants were determined (Table 1). In completely similar experiments where instead of adding ouabain the efflux medium was changed to K-free saline after 7 min, the K-sensitive rate constant was calculated (Table 1).

Effect of ouabain on $[Na]_i$ and net Na influx

Vessels were equilibrated in [^{22}Na]saline at 37 °C and then transferred either to [^{22}Na]saline containing 1 mM-ouabain or to a new solution of [^{22}Na]saline. Fig. 7 shows the $[Na]_i$ of vessels which had been exposed to ouabain for various times, as

well as the $[Na]_i$ of the control vessels. Ouabain exposure caused at first a rapid increase in $[Na]_i$ with a rate constant of 0·21 min^{-1} over the first 2 min. Thereafter the net influx rate decreased: after 2 h $[Na]_i$ was 62 mmol l^{-1} cells.

Because the smooth muscle cell volume was determined before the vessels were incubated in [^{22}Na]saline the results would be influenced if the media volume changed during the prolonged incubations. In twenty experiments the media volume was therefore determined immediately after mounting on the myograph at room temperature, after warming to 37 °C and again after 2 h at 37 °C in either physiological saline (ten vessels) or physiological saline with 1 mM-ouabain (ten vessels). Neither the warming, the physiological saline incubation nor the ouabain incubation caused a significant change in the media volume.

DISCUSSION

$[Na]_i$

The $[Na]_i$ of the vessels used in this study, as determined by the method of Friedman (1974), was 13·7 mmol l^{-1} cells. We cannot, however, exclude the possibility that this estimate includes some extracellular Na which has not exchanged with Li. However, our finding that disruption of the cell membrane reduces our measurements of $[Na]_i$ to under 2 mmol l^{-1} cells indicates that any such extracellular contribution is small. Our method of relating the Na content to cell volume, estimated by light microscopy, has the advantage that it avoids the problems involved in using extracellular markers. On the other hand it raises a number of other problems. First, the cells in the intima and adventitia are not included in the determination. The proportion of cells in the intima and adventitia is however rather small (less than 10%) in these arteries (C. Aalkjær & M. J. Mulvany, unpublished observation), and these cells are therefore unlikely to have caused serious error. Secondly, the muscle cell volume is measured before exposure to the various incubation media. The similarity of the control measurements of media volume before and after incubation, however, showed that this problem is also of minor importance. Thirdly, because it is not possible to determine the fraction of smooth muscle cells in the media of each individual vessel segment, the ratio 0·71 has been used throughout as a mean value for this parameter. This value was determined earlier in this laboratory (Mulvany *et al.* 1978) and has a standard deviation of 0·023, suggesting that 10% of the variance in the determinations of $[Na]_i$ may be attributable to variability of the cellular content of media. Fourthly, the trauma inflicted during dissecton would have a relatively large effect on determination of $[Na]_i$ in these small vessels. However, we found no correlation between vessel size and $[Na]_i$. Therefore, we consider that despite these problems, the $[Na]_i$ determined (13·7 mmol l^{-1} cells) is a good estimate of the $[Na]_i$ in these vessels.

In order to compare this estimate of $[Na]_i$ with the estimates of the Na content of other vessels it is necessary to express our estimate in the units used there. From determinations of morphological parameters in representative vessels we found the dry weight/volume ratio to be 0·33. If the specific gravity of vessels is about 1·06 (Keatinge, 1968), the proportion of solids in the vessel wall is about 0·31. Taking this figure as the proportion of solids in the smooth muscle cells implies that $[Na]_i$ was

about 18 mmol l^{-1} cellular water. The dry weight/smooth muscle cell volume ratio was 0·66 indicating that $[Na]_i$ was about 20 mmol kg^{-1} dry weight. These values are of the same order as those found in rat aorta (12 mmol kg^{-1} cellular water, Jones, 1974) and tail artery (25 mmol kg^{-1} dry weight, Friedman, Mar & Nakashima, 1974; about 11 mmol kg^{-1} cellular water, Garay, Moura, Osborne-Pellegrin, Papadimitriou & Worcel, 1979). They are, however, substantially smaller than those obtained from rabbit portal vein using electron-probe analysis (47 mmol kg^{-1} cellular water, Somlyo, Somlyo & Shuman, 1979). Whether this points to the presence of a temperature-insensitive, rapidly exchanging intracellular Na pool not detectable with our method, or to a difference between arteries and veins, or whether it is due to the different methodology, remains to be determined (Jones, 1982).

Na fluxes

The washout of Na into physiological saline at 37 °C can in larger arteries be split up into three phases (Garrahan, Villamil & Zadunaisky, 1965; Garay *et al.* 1979), where phase 3 represents efflux from a very small compartment. We found, however, only two phases, probably because the method we have used is not sensitive enough to detect the third phase in the small vessels. The first phase is very fast and probably consists of extracellular and extravasal ^{22}Na. The second phase is considered to be an intracellular phase for the following reasons. First, back-extrapolation of this phase gave a value for the zero time intercept of 13·5 mmol l^{-1} cells, which is not different from that found for $[Na]_i$ using the cold Li method. Secondly, about 50 % of the phase is ouabain dependent, which is not different from that expected for the intracellular phase (Garay *et al.* 1979). The main part of the ouabain-resistant efflux is probably a temperature-sensitive efflux, since the efflux in cold Li saline (see Methods) was only about 4 % of the total efflux. The nature of this temperature-sensitive efflux, however, requires further experimental investigation. Thirdly, a 50 % decrease in efflux was also seen when omitting K from the efflux medium, suggesting that 1 mM-ouabain was sufficient to completely inhibit the Na–K-ATPase. Fourthly, the efflux is equivalent to the influx (which would be expected in a steady-state condition). On this basis the intracellular Na efflux rate constant is 0·137 min^{-1} and therefore similar to that found for the intracellular phase in rat tail artery (0·145 min^{-1}, Garay *et al.* 1979), although somewhat smaller than has been reported for aorta (0·18 min^{-1}, Jones, 1974). Since the cell volume/cell area ratio of these vessels is 0·84 μm (Aalkjær & Mulvany, 1983), this corresponds to an efflux rate of 27·3 nmol m^{-2} s^{-1}.

Because the diffusion pathway in these vessels is very small it is possible to compare the acute decrease in Na efflux upon addition of ouabain (Fig. 6) with the acute increase in $[Na]_i$ upon addition of ouabain (Fig. 7). This revealed that the rate constant of the increase in $[Na]_i$ (0·21 min^{-1}) was much higher than expected from the decrease in efflux (0·060 min^{-1}). This indicates that ouabain in this high concentration (1 mM) causes an increase in the Na permeability. This finding could partly be explained by the acute depolarization seen after addition of ouabain (Hendrickx & Casteels, 1974; Mulvany, Nilsson, Flatman & Korsgaard, 1982).

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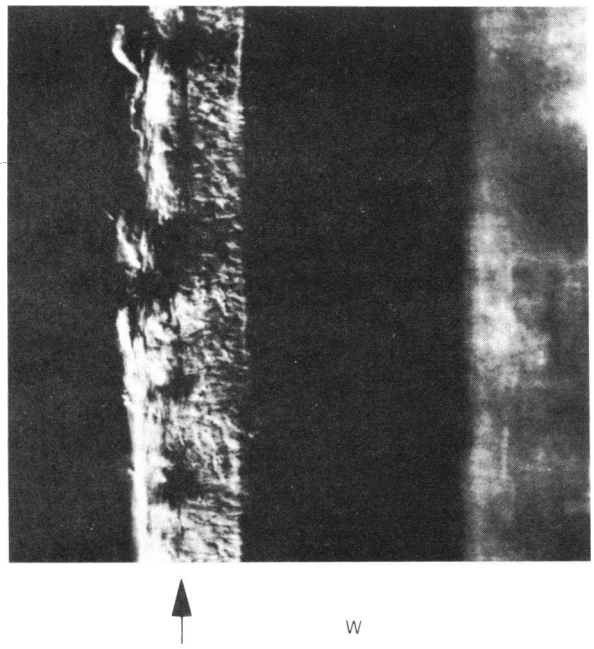
EXPLANATION OF PLATE

Light micrograph of a vessel (internal diameter about 200 μm) mounted in the myograph. The photograph was taken using Normarski interference-contrast microscopy. With the microscope focused on the outer edge of the mounting wire (W) the vessel is seen in longitudinal section. The arrow indicates the adventitia–media border. Segment and media volumes were determined (see text) from measurements of wall thickness, media thickness, internal circumference and segment length.


W