A ^{133}Cs Nuclear Magnetic Resonance Study of Endothelial Na^+-K^+-ATPase Activity: Can Actin Regulate Its Activity?

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ABSTRACT Using $^{133}Cs^+$ NMR, we developed a technique to repetitively measure, in vivo, Na^+-K^+-ATPase activity in endothelial cells. The measurements were made without the use of an exogenous shift reagent, because of the large chemical shift of 1.36 ± 0.13 ppm between intra- and extracellular Cs^+. Intracellularly we obtained a spin lattice relaxation time (T_1) of 2.0 ± 0.3 s, and extracellular T_1 was 7.9 ± 0.4 s. Na^+-K^+ pump activity in endothelial cells was determined at 12 ± 3 nmol $Cs^+ \cdot min^{-1} \cdot (mg\ Prot)^{-1}$ under control conditions. When intracellular ATP was depleted by the addition of 5 mM 2-deoxy-D-glucose (DOG) and NaCN to about 5% of control, the pump rate decreased by 33%. After 80 min of perfusion with 5 mM DOG and NaCN, reperfusion with control medium rapidly reestablished the endothelial membrane Cs^+ gradient. Using $^{133}Cs^+$ NMR as a convenient tool, we further addressed the proposed role of actin as a regulator of Na^+-K^+ pump activity in intact cells. Two models of actin rearrangement were tested. DOG caused a rearrangement of F-actin and an increase in G-actin, with a simultaneous decrease in ATP concentration. Cytochalasin D, however, caused an F-actin rearrangement different from that observed for DOG and an increase in G-actin, and cellular ATP levels remained unchanged. In both models, the Na^+-K^+-pump activity remained unchanged, as measured with ^{133}Cs NMR. Our results demonstrate that ^{133}Cs NMR can be used to repetitively measure Na^+-K^+-ATPase activity in endothelial cells. No evidence for a regulatory role of actin on Na^+-K^+-ATPase was found.

INTRODUCTION

Vascular endothelium plays an important role in the regulation of vascular function, especially the regulation of flow-mediated arterial tone. In vitro studies have demonstrated that endothelial cells are dynamically responsive to shear stress, caused by flowing blood (Bodin et al., 1991). As a result, these cells show transient or long-lasting modifications in their structure and function. The extent to which these changes involve changes in ion homeostasis is still unknown. Olsen et al. (1988) demonstrated that shear stress induced a K^+-selective ionic current in endothelial cells. This K^+ current hyperpolarizes the plasma membrane, causes an enhanced Ca^{2+} inflow, and alters the ionic balance. Because of a change in the Na^+/K^+ gradients, a change in the Na^+-K^+-ATPase activity occurs. For instance, an increase in intracellular Na^+ increases the pump activity. Several ion transport systems in cultured endothelial cells have been described; among these are the Na^+-K^+-Cl^- cotransporter, the Na^+-H^+ exchanger, and Na^+-K^+-ATPase (Brock et al., 1986). All of these transport systems are vital for the maintenance of cell volume and membrane potential. Sodium and potassium gradients are generated and maintained by the activation of the Na^+-K^+ pump and used by other ions and molecules for gradient-driven transport across the cell membrane. For this reason we developed a NMR technique to study such changes before and during cellular interventions causing changes in Na^+-K^+-ATPase activity.

Because of its low sensitivity and the associated poor temporal resolution, $^{39}K^+$ NMR has a major disadvantage compared with its biological congener, $^{87}Rb^+$. On the other hand, $^{87}Rb^+$ NMR results in large line widths and, like $^{39}K^+$, has, in biological systems, isochronous resonances for intra- and extracellular ions (Shehan et al., 1993). Only with relatively large amounts of shift reagents is one able to observe those resonances separately (Allis et al., 1989). As high shift reagent concentrations disturb the Ca^{2+} distribution, $^{87}Rb^+$ NMR may not be a good choice to study K^+ fluxes in endothelial cells. Davis et al. showed that $^{133}Cs^+$ NMR could be used to study K^+ fluxes in erythrocytes and rat hearts (Davis et al., 1988). Cesium, present in low concentrations in perfusates, was reported not to seriously compromise the mechanical and electrical properties of the heart. Furthermore, endothelial cells do not show different electrochemical, transport, and distribution properties for Cs^+ as compared to K^+ (De Smet et al., 1994). These electrochemical studies showed that replacement of K^+ with Cs^+ resulted in similar reversal potential and time course of activation under osmotic stress. Although solvated Cs^+ has 40% more volume than K^+ (Hahn, 1988), this volume difference does not seem to interfere with recognition by, for instance, Na^+-K^+-ATPase as the substrate. However, replacement of K^+ by Cs^+ has been reported to result in a lower Na^+-K^+-ATPase activity in purified enzyme extracts from nerve cells (Skou, 1960).

Previously we reported on the determination of Na^+-K^+-ATPase activity in cultured vascular endothelial cells grown

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on microcarrier beads using ^{23}Na NMR (Gruwel et al., 1995). In those studies the pump activity was determined by inhibiting the pump with ouabain while recording the increase in intracellular Na^+. From the initial rate of Na^+ uptake, the pump activity was determined. Unfortunately, only one such determination per cell batch was possible because the inhibitor, ouabain, could not be successfully washed out. A less potent analog of ouabain was not used.

The Na^+-K^+ pump activity can be regulated through changes in its substrates, for instance, ATP concentration. Recently it was proposed that its activity could also be regulated through other pathways, such as pump phosphorylation and actin binding. Bertorello and Katz postulated for epithelial cells a regulation of pump activity through short actin filaments (Bertorello and Katz, 1993). Recently Cantiello provided evidence for a stimulating effect of short actin filaments on the Na^+-K^+-ATPase in purified enzyme extracts (Cantiello, 1995). For endothelial cells such a mechanism could be important, because 16–18% of their protein consists of contractile filaments (Schnittler et al., 1992). If the hypothesis were correct, an increase in the cytosolic short-chain actin filaments should increase the Na^+-K^+ pump activity (Bertorello and Katz, 1993). Cytochalasin D was reported to stimulate the activity of purified Na^+-K^+-ATPase when incubated with purified G-actin (Bertorello and Cantiello, 1992). By using $^{133}Cs^+$ NMR and cytochalasin D, which is known to favor the formation of actin oligomers and prevents the formation of long polymers (Sampath and Pollard, 1980; Goddette and Frieden, 1986), this postulate was tested on intact endothelial cells.

MATERIALS AND METHODS

Cell culture and perfusion

Macrovascular endothelial cells were harvested from porcine aortae obtained from slaughterhouse material. After excision from the animals, the aorta segments were rapidly immersed in a phosphate-buffered saline (PBS) solution with added aclocillin (0.2 $g \cdot l^{-1}$) and transported to the laboratory. Endothelial cells were obtained by scraping the inner surface of the aortae with a scalpel. The accumulated material was suspended in culture medium M199 (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany) with, in addition, 10% newborn calf serum (NCS) and 1% penicillin-streptomycin (50 IU $\cdot$ ml^{-1}/50 $\mu g \cdot ml^{-1}$) and 1% amphotericin B (2.5 $\mu g \cdot ml^{-1}$) (Gibco BRL). The endothelial cells were grown on microcarrier beads (Nunc Biosilon, Roskilde, Denmark) with a particle diameter of 160–300 μm. When the cells reached confluence, 1 g carrier contained about $50 \cdot 10^6$ cells. Details of the cell culture have been described previously in detail (Gruwel et al., 1995).

Two perfusion media were used, a K^+ and a Cs^+ medium. The K^+ medium consisted of the following substances (Merck, Darmstadt, Germany) (in mM): (150) NaCl, (2.7) KCl, (1.2) KH_2PO_4, (1.2) $MgSO_4 \cdot 7H_2O$, (1.0) $CaCl_2 \cdot 2H_2O$, (5.1) glucose, (0.5) creatin, (10) HEPES, and 10 vol% NCS, with the pH adjusted to 7.4. For $^{133}Cs^+$ NMR spectroscopy, a Cs^+ medium was used that was identical to the K^+ medium, but with KCl replaced by CsCl. After insertion of the sample in the magnet, the cells were continuously perfused at a rate of 1 $ml \cdot min^{-1}$. Before the start of experiments, the cells were allowed to adapt to 37°C after being held at room temperature for approximately 10 min. This equilibration period took 30 min on average.

Metabolic processes were inhibited by the addition of NaCN and 2-deoxy-D-glucose (DOG) directly to the media before perfusion, resulting in a final concentration of 5 mM for both reagents. Cytochalasin D was added to the perfusate from a dimethyl sulfoxide (DMSO) stock solution, resulting in a final concentration of 1 μM.

Actin filament distribution was studied using cells grown on microscope slides coated with 1% gelatin. After 3–4 days the slides were washed with PBS and fixed with 3.7% paraformaldehyde for 20 min, followed by 5 min of treatment with 0.1% Triton X-100. We assumed that these treatments did not affect the pump activity. Thereafter, cells were treated for 2 h with control medium, medium containing DOG, or cytochalasin D. Subsequently, the cells were washed and incubated for 30 min with rhodamin-phalloidine for F-actin staining or FITC-labeled DNase for G-actin staining (both from Molecular Probes, Eugene, OR). After the final wash, the slides were covered with Slow-Fade (Molecular Probes), a reagent added to suppress photobleaching of the fluorescence. Photographs were taken through a Zeiss Axiovert 35 microscope using 400 ASA black and white film.

All chemicals were purchased from Sigma (St. Louis, MO) unless mentioned otherwise and were of analytical grade.

NMR experiments

The NMR perfusion experiments were performed in a 10-mm NMR tube that could hold 0.84-g carrier beads in the NMR-sensitive volume (Gruwel et al., 1995). All $^{133}Cs^+$ NMR experiments were performed at 52.48 MHz on a Bruker AMX 400 broadband variable temperature probe with 22.5-μs $\pi/2$ pulse lengths. The temperature within the probe could be controlled to within ±0.3°C by using a Bruker Eurotherm K temperature controller. After switching to Cs^+ medium, 4096 data points were accumulated every 10 min in 64 scans to follow the Cs^+ accumulation. Before Fourier transformation an exponential line broadening of 5 Hz was applied to the free induction decay. To calculate intracellular Cs^+ concentrations, an external calibration capillary containing a known Cs^+ concentration was always included in the experimental set-up. The Cs^+ resonance of the calibration capillary was shifted 47.5 ppm downfield. To calculate the intracellular Cs^+ concentration, an endothelial cell volume of 3.8 $\mu l \cdot (mg\ Prot.)^{-1}$ was used (Gruwel et al. 1995). Spin-lattice relaxation rates were measured with an inversion recovery technique using cyclops phase cycling. Typically 10 time delays were measured, Fourier transformed, and fitted to a single-exponential, three-parameter expression. Nonlinear regression analyses of the data were performed with Sigmaplot (Jandel Scientific, Corte Madera, CA), which contained the Marquardt-Levenberg algorithm.

Theory

The Na^+-K^+-ATPase activity could be determined from a Cs^+ loading experiment. The time dependence of the intracellular Cs^+ signal can be written as

$$\frac{d}{dt}[Cs^+]_i = V_{Cs^+} + k_1[Cs^+]_e - k_2[Cs^+]_i, \qquad (1)$$

describing Cs^+ transport in terms of active and passive fluxes. $[Cs^+]$ represents the Cs^+ concentration, with intra- and extracellular Cs^+ indicated by the subscripts i and e, respectively. The active term, V_{Cs^+} represents the Na^+-K^+ pump rate in $mol \cdot l^{-1} \cdot s^{-1}$. Passive transport is described by the last two terms on the right-hand side of Eq. 1. Here k_1 and k_2 represent the rate constants for passive flux into and out of the cells, respectively. Assuming k_1 to be equal to k_2, Eq. 1 can be solved for $[Cs^+]_i$ by using the boundary condition $[Cs^+]_i(t = 0) = 0$. We obtained

$$[Cs^+]_i(t) = \frac{V_{Cs^+} + k[Cs^+]_e}{k} \cdot (1 - \exp(-k \cdot t)). \qquad (2)$$

For $t \to \infty$ one observes that $k \cdot [Cs^+]_i(\infty) = V_{Cs^+} + k \cdot [Cs^+]_e$. Thus for

the Na^+-K^+-ATPase activity one can write

$$V_{Cs^+} = k \cdot ([Cs^+]_i(\infty) - [Cs^+]_e). \quad (3)$$

As $[Cs^+]_i(\infty) \gg [Cs^+]_e$, the following approximation can be made:

$$V_{Cs^+} \approx k \cdot [Cs^+]_i(\infty). \quad (4)$$

Allis et al. obtained a similar expression for the Na^+-K^+-ATPase activity.

The experimentally obtained Cs^+ accumulation curves were fitted to a three-parameter expression, $[Cs^+]_i(t)$ = constant + $[Cs^+]_i(\infty) \cdot (1 - \exp(-k \cdot t))$, resembling Eq. 2. The constant was used to check for any baseline offset, which was always smaller than 0.1% of the signal amplitude after complete Cs^+ loading. From the fit the Na^+-K^+ pump activity was determined as described by Eq. 4.

RESULTS

Fig. 1 shows a Cs^+ NMR spectrum obtained after complete Cs^+ loading of the cells. Without the use of an extrinsic shift reagent, a chemical shift of 1.36 ± 0.13 ppm (n = 10) between the intra- and extracellular resonance was obtained. The intracellular line width at half-height was 30 ± 4 Hz (n = 10). After 2.5 h of perfusion with Cs^+ medium, the intracellular Cs^+ signal intensity remained stable. From the integral of this Cs^+ resonance the intracellular Cs^+ concentration, $[Cs^+]_i(\infty)$, could be determined, after calibration using a capillary with a known amount of Cs^+. We assume that the endogenous shift reagent does not affect the NMR visibility (Shehan et al. 1993). An intracellular Cs^+ concentration of 148 ± 12 mM (n = 10) was found (vide infra). At this point in time the Cs^+ spin-lattice relaxation time (T_1) was measured. Intracellularly we obtained a value of 2.0 ± 0.3 s (n = 4), and extracellularly T_1 = 7.9 ± 0.4 s (n = 4). The experimental recycle time was selected to allow enough time for spin-lattice relaxation of the magnetization back to equilibrium.

Endothelial cells could repetitively be loaded with Cs^+, as shown in Fig. 2. Washout of intracellular Cs^+ was accomplished using K^+ medium. Both the Cs^+ loading and wash-out periods were restricted to 80 min. After the perfusion with Cs^+ was halted, the cells were perfused with K^+ containing medium. The start of the acquisition of washout data was delayed by 7 min to flush the perfusion lines. To determine accurate pump activities using this type of experiment, data acquisition over a longer time period is required (see Fig. 3). Microcalorimetry experiments (Gruwel et al., 1995) showed no change in the cellular heat flux upon changing K^+ medium to Cs^+ medium (data not shown), indicating no change in the endothelial ATP metabolism. Fig. 3 shows a typical Cs^+ loading experiment. The NMR experiment, collecting consecutive data files every 10 min, was started as soon as the cells were perfused with Cs^+ medium. Each file was Fourier transformed, and the intensity of the intracellular signal was plotted as a function of time in Fig. 3 *A*. The signal intensities were fitted to Eq. 2, and according to Eq. 3 the Na^+-K^+-ATPase activity was calculated. We obtained a pump rate of 12 ± 3 nmol $Cs^+ \cdot min^{-1} \cdot (mg\ Prot)^{-1}$ (n = 10). Inhibition of the Na^+-K^+-ATPase with 50 μM ouabain added to the Cs^+ medium after Cs^+ loading resulted in a passive Cs^+ transport out of the cells, as shown in Fig. 3 *B*. This confirmed previous Na^+ experiments, which indicated only minimal changes in cell volume (Gruwel et al., 1995), indicating only small changes in intracellular osmolytes, if any. The observed influx of Na^+ ions is thus compensated by the efflux of Cs^+, to maintain cell volume.

A protocol was adapted to deplete the endothelial ATP levels during Cs^+ loading. Porcine aortic endothelial cells were reported to contain 16 ± 1.9 nmol ATP · $(mg\ Prot)^{-1}$ under control conditions (Kuhne et al., 1991; Watanabe et al., 1991). These values were confirmed in our laboratory (16 ± 1.9 nmol ATP · $(mg\ Prot)^{-1}$) using a high-performance liquid chromatography technique (Decking et al. 1994). In Fig. 4 the effect of 5 mM 2-deoxy-D-glucose (DOG) and 5 mM NaCN on the Cs^+ loading of the cells is shown (n = 3). Endothelial ATP levels decreased to ~10 (0.42 mM) and ~5% (0.2 mM) after 5 and 30 min of addition, respectively (Watanabe et al., 1991). The Cs^+ accumulation could be halted and even reversed. This indicates a decrease in net pump activity, resulting in a replacement of intracellular Cs^+ with extracellular Na^+ (Gruwel et al., 1995). After 80 min of perfusion with DOG and NaCN, the cells were reperfused with pure Cs^+ medium. Upon reperfusion, the intracellular Cs^+ signal increased again, suggesting that endothelial cells can survive long periods of metabolic inhibition and are able to quickly regenerate Cs^+/K^+ homeostasis during reperfusion. Perfusion with 20 mM DOG alone had no effect on Cs^+ uptake (not shown). Inhibition of glycolytic ATP production, by using 20 mM DOG, did deplete the energy reserves, e.g., ATP (^{31}P NMR, unpublished results); however, the intracellular ATP concentration did not vanish. ^{31}P NMR experiments showed a decrease in the β-ATP signal by 90% (corresponding to ~0.4 mM) after 3 h of perfusion. Watanabe et al. determined an endothelial ATP concentration of ~60% and ~30% in reference to control after, respectively, 15 and 60 min of treatment with 20 mM DOG (Watanabe et al., 1991). This corresponds to a cellular ATP concentration of ~2.6

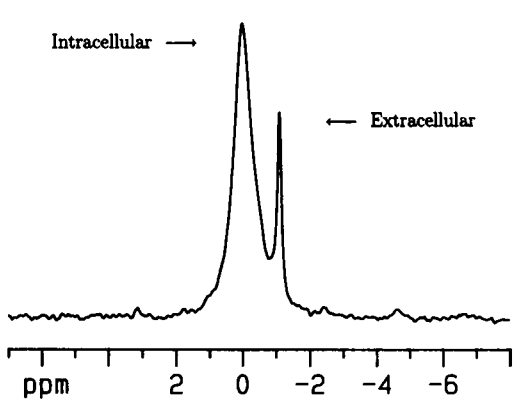

FIGURE 1 $^{133}Cs^+$ NMR spectrum of endothelial cells. $^{133}Cs^+$ NMR spectrum of endothelial cells grown on micro carrier beads after 2.5 h of perfusion with Cs^+ medium.

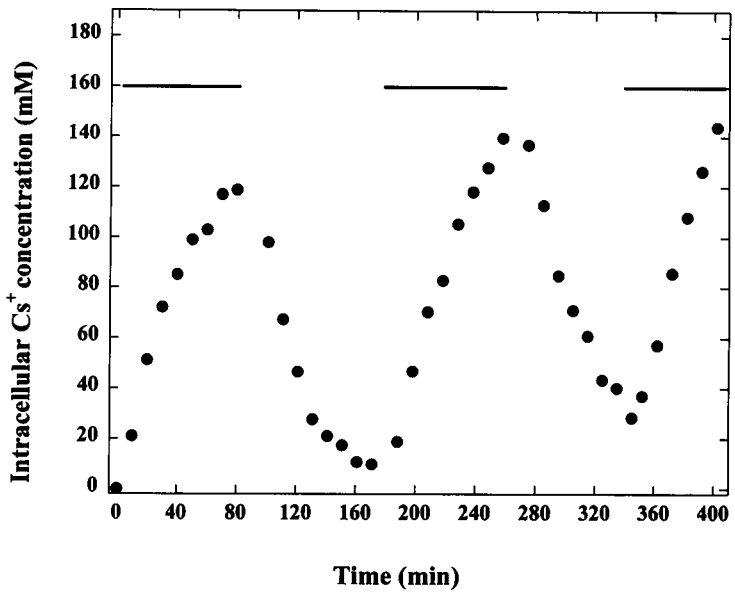

FIGURE 2 $^{133}Cs^+$ NMR signal intensity as a function of time in endothelial cells. $^{133}Cs^+$ NMR of Cs^+ uptake and washout in endothelial cells. The black lines in the figure indicate a period of Cs^+ perfusion. During perfusion with medium containing Cs^+, the intracellular Cs^+ signal amplitude increased. Between the periods marked by the black lines, the cells were perfused with K^+ medium to wash out intracellular Cs^+.

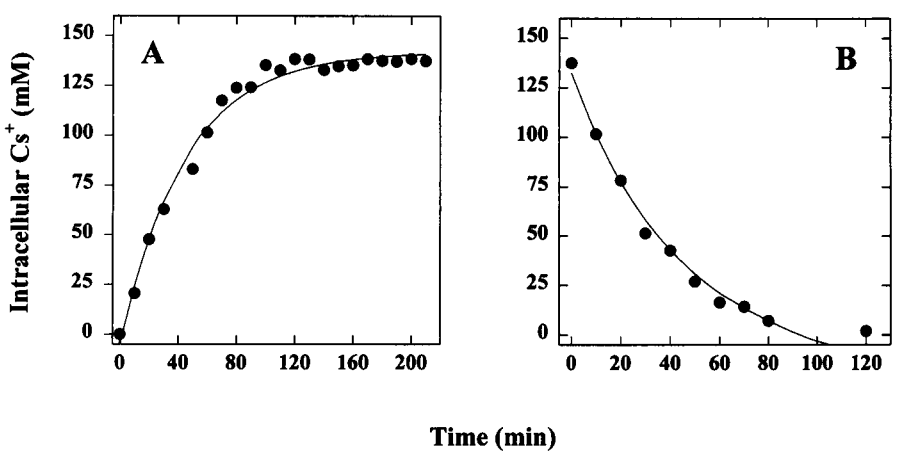

FIGURE 3 $^{133}Cs^+$ loading and washout in endothelial cells. (*A*) A representative Cs^+ loading experiment with fit of the data to Eq. 2. The conversion from concentration (in mM) to amount of Cs^+ (in nmol · (mg Prot)$^{-1}$) can be made using the cell volume of 3.8 μl · (mg Prot)$^{-1}$. (*B*) Intracellular Cs^+ signal during perfusion with 50 μM ouabain. The data were fitted to Eq. 6.

and ~1.3 mM, respectively. This allowed the Na^+-K^+-ATPase to maintain its activity. Microcalorimetry experiments performed in our laboratory showed that cells perfused with medium that contained no glucose and 10 mM DOG reduced their heat production by 75% compared to controls (unpublished results). Although DOG treatment did not affect the pump activity, it caused significant changes in cell morphology, F-actin, and G-actin distribution (Fig. 6, *B*, *E*, and *H*). The amount of G-actin increased at the expense of F-actin. However, when using 5 mM NaCN by itself to block oxidative phosphorylation, we did not observe a decrease in Na^+-K^+ pump activity (data not shown).

Perfusing endothelial cells with 1 μM cytochalasin D while recording the $^{133}Cs^+$ NMR spectra did not show a change in pump rate in comparison with the control experiment (n = 3). One representative experiment is shown in Fig. 5, where both control and cytochalasin D perfusion are plotted, for convenience, as starting at time zero, although they were acquired consecutively. Although we were unable to detect an effect on the pump activity, cytochalasin D did cause dramatic changes in cell morphology that could be described as cell shrinkage and a rounding-up of the cells (Fig. 6 *C*). As can be seen from the data presented in Fig. 6, there is a pronounced redistribution of F-actin (Fig. 6 *F*) and an increase in the concentration of G-actin (Fig. 6 *I*) in endothelial cells upon the addition of cytochalasin D. A similar distribution pattern was observed with 10 mM DOG (see Fig. 6, *E* and *H*). Treatment with DOG and cytochalasin D caused a complete disappearance of the cortical F-actin ring, which is thought to be physically associated with Na^+-K^+-ATPase (Bertorello and Katz, 1993) (Fig. 6, *D–F*). Still, the patterns of F-actin distribution were clearly different (Fig. 6, *E* and *F*). With cytochalasin D, the loss of actin bundles appears more prominent than for DOG-treated cells. In contrast to DOG treatment, which was accompanied by a reduction in intracellular ATP, cytochalasin D did not change the ATP concentration (^{31}P NMR, not shown). In separate experiments we also tested the effect of cytochalasin D on the cellular heat production, which is a measure of ATP turnover, as previously described (Gruwel et al., 1995).

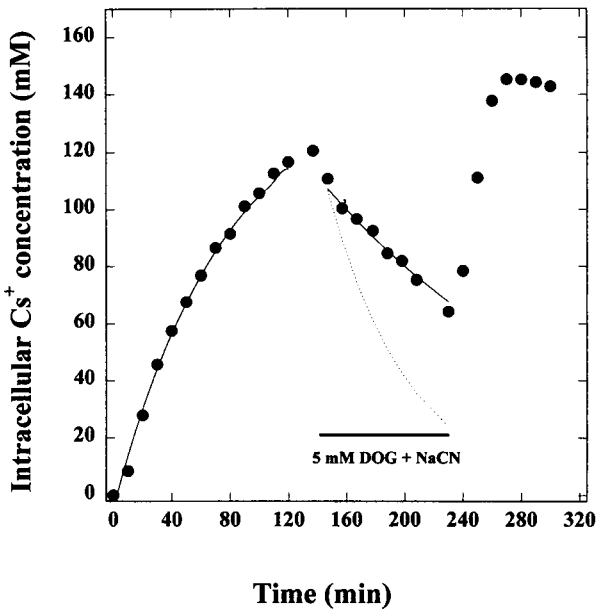

FIGURE 4 $^{133}Cs^+$ signal intensity during Cs^+ loading and ATP depletion. $^{133}Cs^+$ NMR of Cs^+ loading during ATP depletion with 5 mM DOG and 5 mM NaCN. The solid lines represent curves fitted to the data points (see Fig. 3), and the dotted line represents the maximum Cs^+ outflux as calculated from pump inhibition experiments (see Fig. 3 *B*).

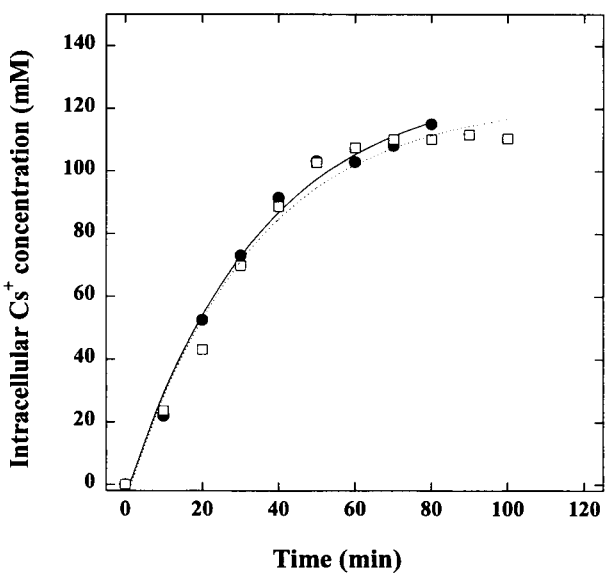

FIGURE 5 Determination of Na^+-K^+ pump activity under control and cytochalasin D perfusion. $^{133}Cs^+$ NMR of Cs^+ loading during control perfusion (□, · · ·) and perfusion with 1 μM cytochalasin D (●, ——). The data sets were plotted as starting at time zero, although they were acquired consecutively using the same sample of carriers and cells. After Cs^+ loading in the control experiment, Cs^+ was washed out using medium containing K^+. After the washout, an experiment with Cs^+ and cytochalasin D was started.

These microcalorimetry experiments showed only a small, immediate (within 10 min), and reversible (within 20 min after return to control perfusion) decrease in heat production of ~10 ± 2% (n = 6) upon treatment with cytochalasin D.

DISCUSSION

The observed chemical shift of 1.36 ± 0.13 ppm at 37°C between intra- and extracellular Cs^+ in endothelial cells is in the range of 0.02–1.76 ppm found for different tissues in cesium-fed rats (Shehan et al., 1993). This shift difference allowed us to observe intra- and extracellular Cs^+ without the use of a shift reagent. Wittenkeller et al. showed with in vitro studies that free 2,3-diphosphoglycerate (2,3-DPG) contributes to the Cs^+ chemical shift (Wittenkeller et al., 1992). Cesium was also shown to exhibit a 2,3-DPG concentration-dependent chemical shift in erythrocytes (Shehan et al., 1993). Although endothelial cells do not contain 2,3-DPG, endothelial cells produce most of their ATP through glycolysis and show the presence of phosphodiesters in their ^{31}P NMR spectra (Decking et al., 1992). It is likely that the observed Cs^+ chemical shift in endothelial cells is caused by the presence of these phosphodiesters. As pointed out by Shehan et al., small concentrations of 2,3-DPG already give rise to a complete shift of the Cs^+ resonance at 302K (Shehan et al., 1993). Consistent with this interpretation is that in rat brain, with known low levels of phosphodiesters, a chemical shift of only 0.02 ± 0.05 ppm has been reported (Shehan et al., 1993).

Vascular endothelial cells contained an average of 10.5 ± 2.4 mg Prot · (gcarrier beads)$^{-1}$, which corresponded to a total intracellular volume of 33 μl within the NMR-sensitive volume (Gruwel et al., 1995). By using this value, the intracellular Cs^+ concentration of 148 ± 12 mM (n = 10) was calculated from the integral of the NMR signal after scaling of the intracellular resonance with the signal of the capillary containing a known amount of Cs^+. The value of 148 mM Cs^+ corresponds well with the known intracellular K^+ concentration.

A minor disadvantage of Cs^+ NMR is the long spin-lattice relaxation time of this ion. This results in a lower temporal resolution as compared to ^{87}Rb NMR. However, for our purpose, the study of Na^+-K^+-ATPase activity, the obtained time resolution allows accurate determination of pump activity. The intracellular T_1 of 2.0 ± 0.3 s is similar to that found in rat muscle and brain tissue (Shehan et al., 1993). Using $^{23}Na^+$ NMR and a shift reagent in combination with microcalorimetry, we recently reported on the energy expenditure of endothelial Na^+-K^+-ATPase (Gruwel et al., 1995). These measurements required inhibition of the pump. In our experiments we used ouabain, which could not be washed out, and this resulted in termination of the experiment. The potential of $^{133}Cs^+$ NMR in Na^+-K^+ pump studies is that it allows one to do multiple experiments on the same cell sample (see Fig. 2) by simply washing out the accumulated Cs^+ after determination of the rate of Cs^+ accumulation. Repetitive measurement of pump activity is therefore possible.

Cs^+ accumulation under control conditions can be used to obtain the Na^+-K^+ pump activity. The pump rate was defined as the slope of the accumulation curve of Fig. 3 *A* at time t = 0 s. From a fit of the data in Fig. 3 *A* to Eq. 2,

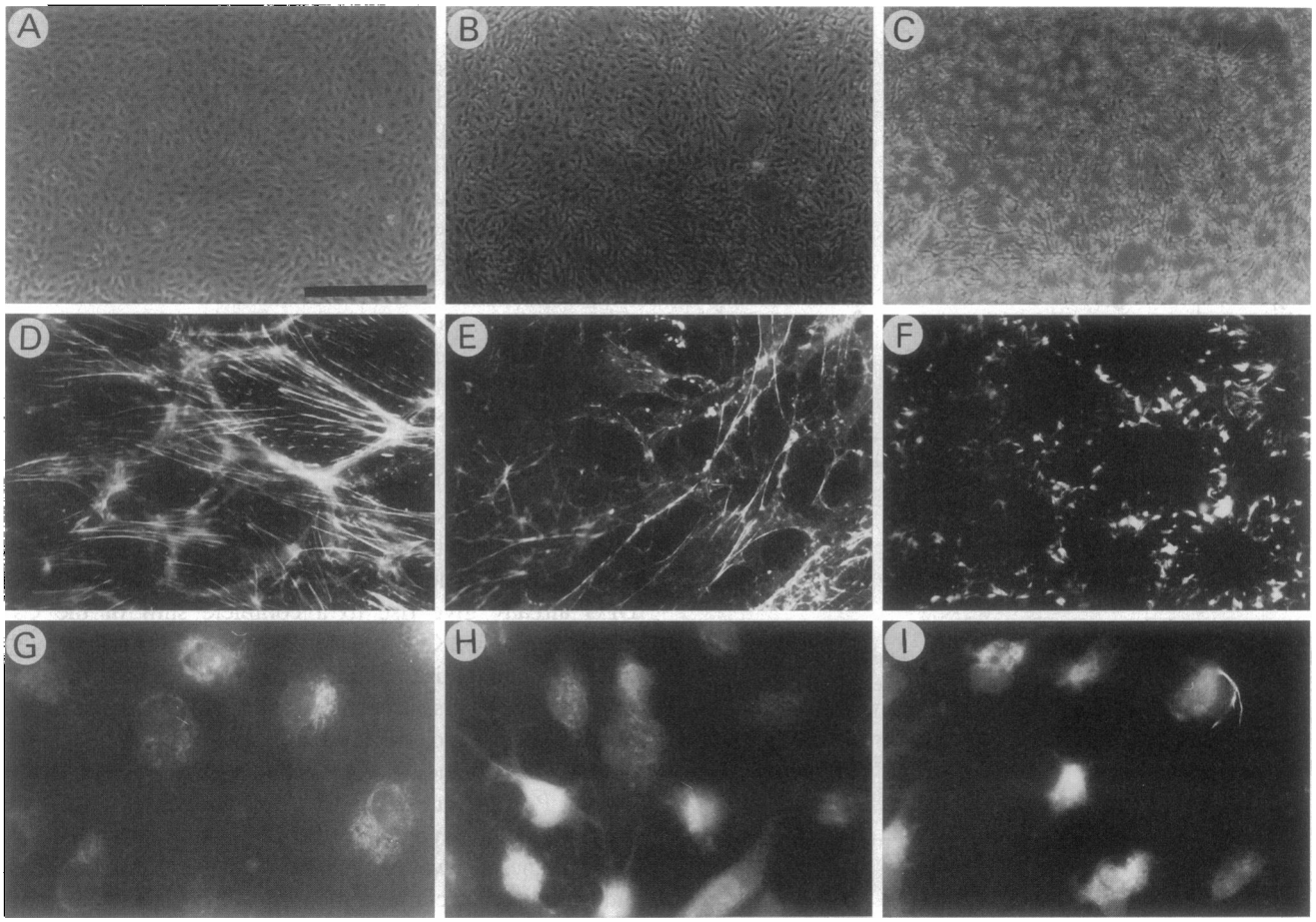

FIGURE 6 Influence of DOG and cytochalasin D on endothelial cell morphology, F- and G-actin distributions. Fluorescence photographs of F-actin distribution, determined by rhodamin-phalloidine staining, and G-actin concentration, using FITC-DNase, in confluent endothelial cells. See Materials and Methods for experimental details. *A*,*D*, and *G* represent the control, untreated cells. (*A*) Cell morphology; (*D*) stained for F-actin; (*G*) stained for G-actin. Experiments with DOG are shown in *B*, *E* and *H*. (*B*) Cell morphology; (*E*) stained for F-actin; (*H*) stained for G-actin. Cells treated with 1 μM cytochalasin D are shown in *C*, *F*, and *I*. (*C*) Cell morphology; (*F*) stained for F-actin; (*I*) stained for I-actin. The bar in *A* represents 100 μm (for *A–C*) and 10 μm (for *D–I*).

$[Cs^+]_i(\infty)$ and k were obtained. The product of these parameters represents the pump rate at $t = 0$. It should be noted that the Cs^+ medium still contained some K^+, because only KCl was replaced with the corresponding cesium salt, whereas KH_2PO_4 was not. As a result, the value for $[Cs^+]_i(\infty)$ as obtained from the fit must be replaced by

$$[Cs^+]_i(\infty)|_{(true)} = [Cs^+]_i(\infty)|_{(fit)} \cdot \frac{[K^+](\text{in } K^+ \text{ medium})}{[Cs^+](\text{in } Cs^+ \text{ medium})}. \quad (5)$$

This assumes that Cs^+ and K^+ are transported inward in the same way. After this correction the Cs^+ pump rate was determined to be 12 ± 3 nmol $Cs^+ \cdot min^{-1} \cdot (mg\ Prot)^{-1}$. This value compared well with the Na^+ pump rate of 17 ± 3 nmol $Na^+ \cdot min^{-1} \cdot (mg\ Prot)^{-1}$ obtained from recent ^{23}Na NMR experiments (Gruwel et al., 1995). The ratio of the two transport rates is in excellent agreement with the known pump stoichiometry of Na^+:K^+ = 3:2 per ATP used. However, care should be taken with the interpretation of this result. Cesium has been reported to be transported by Na^+-K^+-ATPase extracts with different, slower kinetics than K^+, as was shown in ATP hydrolysis experiments (Skou, 1960). In addition, de Smet et al. report Cs^+ to be a good electrochemical congener of K^+ in endothelial cells (de Smet et al., 1994).

Pump inhibition with 50 μM ouabain resulted in a steady decrease of intracellular Cs^+ in preloaded endothelial cells (Fig. 3 *B*). Using an adjusted Eq. 2,

$$[Cs^+]_i(t) = [Cs^+]_i(\infty) \cdot \exp(-k \cdot t), \quad (6)$$

the Cs^+ flux out of the cells could be obtained. The rate at which Cs^+ was expelled from the cell was, within experimental error, equal to the accumulation rate obtained from Fig. 3 *A*.

Endothelial cells are characterized by a mainly glycolytic energy metabolism, as they are essentially not dependent on a constant oxygen supply in the presence of 5 mM glucose (Kuhne et al., 1991). Under control conditions porcine aortic endothelial cells contain 16 ± 1.9 nmol ATP · $(mg\ Prot)^{-1}$ (Kuhne et al., 1991; Watanabe et al., 1991). Using

a cell volume of 3.8 μl · (mg Prot)$^{-1}$ (Gruwel et al., 1995), one calculates an intracellular ATP concentration of 4.2 mM. To deplete the endothelial ATP level, one must inhibit both glycolytic and oxidative pathways of ATP production. Inhibition of oxidative phosphorylation with 5 mM NaCN does not affect significantly the cellular ATP levels within 1 h of addition (<5%) (Watanabe et al., 1991), and a change in pump activity is thus not expected. This was confirmed by our $^{133}Cs^+$ NMR experiments showing no decrease in the Na^+-K^+ pump rate when perfused with 5 mM NaCN as compared with control conditions. Similarly, inhibition of glycolysis with DOG did not result in a change in pump activity. However, a combination of 5 mM DOG and 5 mM NaCN caused the cellular ATP reserves to deplete to ~5% of control, corresponding to a cellular concentration of 0.22 mM. When the cells were perfused with $^{133}Cs^+$ and DOG + NaCN, an immediate reversal of Cs^+ transport was observed (Fig. 4). The observed outward rate was 33 ± 4% of the Cs^+ outflux observed in the presence of 50 μM ouabain. Most likely, ATP was the limiting substrate under these conditions. Under identical experimental conditions Watanabe et al. reported endothelial ATP levels to be as low as 0.2–0.5 mM (Watanabe et al., 1991). This value compares well with the reported in vitro data on the K_m for Na^+-K^+-ATPase. Binding constants have been reported for rat brain cells (K_m(ATP) ≈ 0.5 mM) (Robinson, 1976), rat vascular smooth muscle cells (K_m(ATP) = 0.83 mM) (Aviv et al., 1993), and dog tracheal epithelium (K_m(ATP) = 0.4 mM) (Westenfelder et al., 1980). These data, together with the pump inhibition observed in this study, suggest an apparent in vivo K_m(ATP) of $0.22 \leq K_m(ATP) \leq 1.3$ mM for the Na^+-K^+ pump in porcine endothelial cells. Note that with 20 mM DOG, resulting in a cellular ATP concentration of 1.3 mM, we observed no effects on the pump rate. It also demonstrates that drastic measures must be applied to deplete cellular ATP sufficiently and cause measurable pump inhibition. Furthermore, the experiments with DOG suggest that glycolytically produced ATP has no specific access to the Na^+-K^+ pump arguing against metabolic compartmentation of ATP in endothelial cells. During reperfusion with control medium in Fig. 4, we observed a rapid influx of Cs^+. This influx occurred at a rate larger than that observed during controls. This could be explained by a stimulation of the Na^+-K^+ pump resulting from an increased intracellular Na^+ concentration. It is known that a small increase in the intracellular Na^+ concentration in endothelial cells causes an increase in pump rate up to a factor of 2 (Gruwel et al., 1995).

This study further tested the proposed regulatory role of small actin filament on Na^+-K^+-ATPase (Cantiello, 1995; Bertorello and Katz, 1993) by combining two different techniques: 1) Cs^+ NMR and 2) fluorescence microscopy to cytochemically determine F- and G-actin. Bertorello and Katz postulated that an increase in the concentration of short actin filaments could regulate the pump (Bertorello and Katz, 1993). Recently Cantiello showed that short actin filaments, obtained from rabbit muscles, did stimulate the Na^+-K^+-ATPase by using purified enzyme from the rat renal cortex (Cantiello, 1995). Although in these experiments the Na^+-K^+-ATPase/actin ratio (1:500 or more) was rather large, it was suggested that such large ratios may even occur in vivo if one accounts for the cell surface-to-volume ratio and the heterogeneity of the actin distribution/concentration in the cytoplasm. Contrary to this prediction, we were unable to find such a stimulation of the Na^+-K^+-ATPase in endothelial cells, known to have a large surface-to-volume ratio, with $^{133}Cs^+$ NMR (see Fig. 5).

Cytochalasin D is known to inhibit actin polymerization (Bertorello and Katz, 1993; Bretscher, 1991) and accordingly promotes the formation of G-actin. Cytochalasin D also stimulates the formation of oligomeric actin filaments through its capping activity of the fast growing actin ends, as well as through filament cutting activity (Bretscher, 1991). Consistent with this action, we observed shortened F-actin filaments and an increase in G-actin concentration (Fig. 6, *A* and *D*). Similar observations were made using DOG. Whereas DOG caused substantial ATP depletion, cytochalasin D did not change the intracellular ATP.

In summary, because we were not able to detect changes in pump activity, despite pronounced cytoskeletal changes, our findings suggest that actin redistribution in intact endothelial cells does not play a significant role in the regulation of Na^+-K^+-ATPase activity in this cell type. Our measurements indicate that Cs^+ NMR can be used as a powerful tool in the study of short-term regulation of Na^+-K^+-ATPase in vitro.

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