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Cation Transport in Vascular Endothelial Cells and Aging

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Summary. To understand the generation and maintenance of Na and K gradients in cultured vascular endothelial cells, net Na and K movements were studied. Ouabain-sensitive (OS) net Na gain and K loss were estimated as the difference between the cation content in the presence of ouabain and that in the control. Ouabain- and furosemide-resistant (OFR) fluxes were determined in the presence of the two inhibitors. When the normal medium bicarbonate and phosphate buffers were replaced by N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid both the OS and OFR fluxes decreased more than 50%. Ouabain-sensitive and ouabain- and furosemide-resistant fluxes decreased with increasing cellular age (passage number) an effect not observed when the cation movements were studied in the absence of bicarbonate and phosphate. These results suggest that cultured vascular endothelial cells possess bicarbonate- and phosphate-dependent Na and K pathways which account for a significant portion of their passive movements. Furthermore, the behavior of cation permeabilities with passage number suggests that these modulations may be related to the cellular aging process.

Key Words endothelium · ion transport · net fluxes · aging · bicarbonate · phosphate

Introduction

In recent years, vascular endothelial cells (VEC) have been the focus of biochemical and pharmacological studies to elucidate their important functions as barrier between blood and underlying tissues. Only very recently, a few studies have been published on unidirectional ion fluxes in these cells, suggesting cell volume control by monovalent ion pumps and leak pathways [9, 11, 18]. Ouabain-resistant (OR) leaks for Na and K ions of magnitude equal to or larger than the Na/K pump have been reported in VEC, as in some other cells of apparently normal volume [9, 12, 18–21]. However, no net cation flux studies have been reported as yet which define the ionic homeostasis of VEC, an experimental prerequisite to understand the generation and maintenance of the Na and K gradients in these cells.

In the present work, Na and K transport was determined by net flux measurements which, being equal to the difference between inward and outward ion and water movements, provide accurate information on mass and water transport and thus may provide a basis for further unidirectional studies. The ouabain-sensitive (OS) fluxes were determined as the net Na gain and K loss induced by ouabain. These net movements of Na and K could be considered as a measure of the Na/K pump provided ouabain does not induce *per se* new Na or K pathways. Vascular endothelial cells are known to possess a OR and furosemide-sensitive (FS) Na–K–Cl cotransport [9, 18] which contributes to the leak pathways. The term leak, as used in this study, includes all OR fluxes, i.e., through carriers and channels. Thus, under certain conditions the Na–K–Cl cotransport was inhibited with furosemide to measure the ouabain- and furosemide-resistant (OFR) fluxes.

The results suggest that VEC possess bicarbonate- and phosphate-dependent Na and K pathways which account for a significant portion of the OFR fluxes. Furthermore, the evolution of cation permeabilities with passage number suggests that these modulations are related to the cellular aging process.

Preliminary reports on part of these studies have been published in abstract forms elsewhere [3, 4].

Materials and Methods

Materials

The endothelial clone BFA-1c and cell line BFAE-39 originated from bovine thoracic aorta of normal 6 and 4–5 months old male fetuses, respectively (NIA Aging Cell Repository, Coriell Institute for Medical Research, Camden, NJ, Rep. Nos. GM 3905A and AG 07680, respectively). Media and chemicals used were: minimum essential medium (MEM), F-12 nutrient mixture (Ham) (HF12), cell culture media supplements, trypsin, penicillin, streptomycin, amphotericin B, and ethylene diamine-tetraacetic acid (EDTA) (GIBCO, Grand Island, NY); defined fetal bovine serum

(Hyclone Laboratories, Logan, UT); bovine serum albumin (BSA) (C No. A7030), tris (hydroxymethyl) amino methane base (Tris), 3-[N-morpholine] propane sulfonic acid (MOPS), N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid (HEPES), and ouabain (Sigma Chemical, St. Louis, MO); furosemide (Hoechst-Russel Pharmaceuticals, Somerville, NJ).

Cell Culture

Bovine aortic endothelial cells were propagated in MEM containing 26 mM $NaHCO_3$, 25 mM HEPES and 20% uninactivated fetal bovine serum (FBS) (Strain BFA-1c, passages 3–5, cumulative population doublings, CPD, over 60, [2]) or in HF12 (Strain BFAE-39, passages 3–11, CPD, less than 30) supplemented with antibiotics and 10% fetal bovine serum (FBS). Cells were subcultured with trypsin-EDTA as described elsewhere [2], and passaged at a ½ or ⅕ ratio in 81-cm^2 flasks. For flux measurements, cells were cultured in 35 mm diameter dishes, fed every other day, and used at confluence (4–7 days). The splitting ratios were one 25-cm^2 flask into 15 dishes or one 81-cm^2 flask into 50 dishes.

Cell Cation and Protein Determinations

Na and K were determined by atomic absorption spectrophotometry (Perkin Elmer 5000) and protein content by the method of Lowry et al., as described previously [2].

Measurements of Net Na and K Movements

In this study, poisoning of the Na/K pump with ouabain induced a net Na gain and K loss as a function of time. These changes in Na and K content derive from movements of Na and K through pathways different from the pump, such as channel- and carrier-mediated mechanisms, with the inclusion of the Na–K–Cl cotransport system recently described in BVEC [9].

To measure initial rates, cultures were washed three times at 37°C with 1 ml/dish of either original culture medium (CM) (MEM for clone BFA-1c and HF12 for cell line BFAE-39), or balanced salt solution (BSS) containing (in mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES buffered to pH 7.4 at 37°C with Tris base, and then equilibrated for 10 or 45 min at 37°C in supplemented CM, or 10 mM glucose plus 1 mg/ml BSA in BSS. After the medium was removed, cells were incubated in either supplemented CM or BSS with BSA, and with inhibitors where appropriate. Ouabain and furosemide concentrations were 2 and 1 mM, respectively. Triplicate samples were removed at different time intervals between 0 to 90 min. The flux medium was aspirated, the monolayers were washed with chilled $MgCl_2$ washing solution (in mM: 108 $MgCl_2$, 10 Tris MOPS, pH 7.4 at 4°C, and 5 glucose), and the Na, K and protein contents were determined as described elsewhere [2]. During the equilibration period and flux measurements, the cells were incubated at 37°C in an oven (Precision Thelco, Model 2, Precision Scientific).

In control experiments, the Na and K content and the extracellular pH were measured after cells were removed from the CO_2 incubator and directly incubated, in the same cultured medium (HF12), in an oven with air atmosphere for up to 60 min without washing or equilibration. As expected, evaporation of CO_2 from the medium led to an increase in extracellular pH; however, the Na and K content remained unaltered (Table 1), indicating that the buffer capacity of the cells may have been sufficient to maintain the cation steady state during that period of time.

Table 1. Effect of incubation in nominally CO_2-free atmosphere on cation composition of cultured endothelial cells

Incubation time (min)	Na (nmol/mg protein)	K (nmol/mg protein)	Medium pH
0	45.1 ± 2.6	1,164 ± 59	7.1
10	40.8 ± 2.7	1,296 ± 26	7.2
30	38.9 ± 1.5	1,294 ± 24	7.3
60	46.6 (2)	1,207 ± 23	7.8

Clone BFAE-39, $P_{11}D_5$, $\bar{X}$ ± SEM, $n = 3$, medium HF-12.

In further experiments, the pH of all solutions and culture medium was adjusted with acid or base, at room temperature to give 7.4 at 37°C. This procedure was done in a covered container to reduce evaporation of CO_2. Likewise, the osmolality of all solutions was adjusted to 300 ± 10 mOsM.

The flux rates were expressed in nmol/mg protein · min and were calculated as the slope of the linear function relating net Na gain or K loss with time between 0–90 min. The corresponding slopes were calculated by linear regression analysis. When appropriate, net Na and K movements were determined at one time point for times at which initial rates were still maintained. The slopes were obtained as follows: for the OS fluxes, by subtracting the cell Na and K content in medium containing ouabain from that in the control condition (MEM, HF12, or BSS) and for ouabain- and furosemide-resistant (OFR) fluxes by subtracting the cation content in the presence of ouabain plus furosemide from that in the control condition.

Composition of the Incubation Medium

Table 2 summarizes the buffer composition of each of the six conditions tested in this study.

Statistics

Results are expressed as mean ± SEM ($\bar{X}$ ± SEM). Statistical significance was assessed by Student's one- or two-tailed paired or unpaired *t*-test analysis.

Results

Cellular monovalent cation contents were determined as function of time of incubation of BFA-1c cells in culture medium (MEM) or in balanced salt solution (BSS). In the experiment shown in Fig. 1 the Na and K contents of controls were comparable in MEM and BSS for the time studied. When ouabain (2 mM) was present, the cells gained Na and lost K in a linear fashion over the 60–90 min observation period. The calculated rates of OS net Na gain and K loss were about 100 and 40%, respectively, larger

Table 2. Buffer composition of media for flux measurements

Buffer	Conditions						
	A		B	C	D	E	F
	MEM	HF12	BSS	MBSS (mM)	MBSS	MBSS	MBSS
$NaCHO_3$	26	14	0	0	14	14	14
NaH_2PO_4	1	0	0	0 or 10	0	0	0
Na_2HPO_4	0	1	0	1.9 or 10	0	1.9	1.9
HEPES	25	0	20	0	0	0	0

Culture medium contained all the supplements (serum, vitamins, and amino acids). BSS did not contain supplements but 1 mg/ml of BSA (98–99%) albumin, essentially fatty acid free). MBSS was similar to BSS except for the buffers. Condition F had in addition 0.01 mM $CaSO_4$, 3 mM $FeSO_4$, and 3 mM $ZnSO_4$.

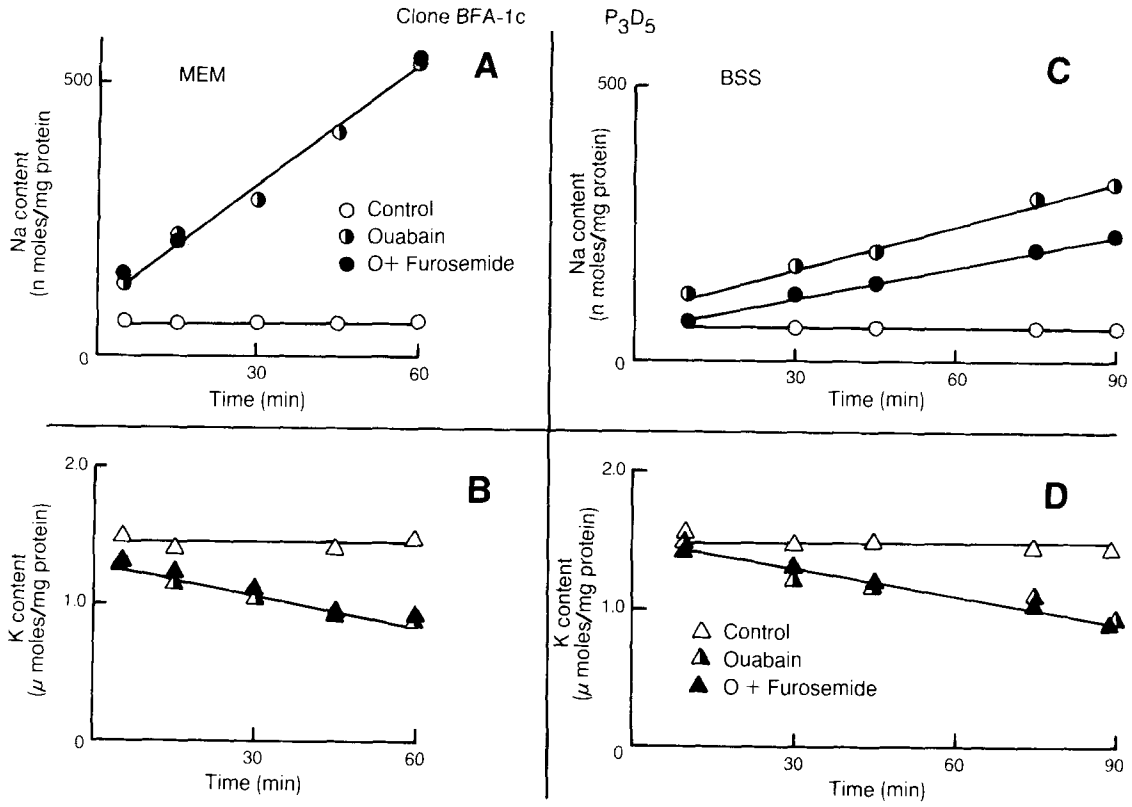

Fig. 1. Na and K content of cultured vascular endothelial cells (CVEC) as a function of time. Clone BFA-1c at passage 3, day 5 (P_3D_5). In the left panel (*A* and *B*), cells were incubated in culture medium (*MEM*), and in the right panel (*C* and *D*) in balanced saline solution (*BSS*). Ouabain and furosemide concentrations were 2 and 1 mM, respectively. Equilibration time was 10 min. Note that Na content is in nmol/mg protein and K content in μmol/mg protein. Slopes were obtained by linear regression

in MEM than in BSS (*compare* Fig. 1*AB* with 1*CD*). Addition of furosemide (1 mM) did not further affect the OS net Na gain or K loss in both media with the exception of the data in panel *C* where furosemide reduced the OS net Na gain. Thus BFA-1c cells apparently lack any significant FS net monovalent cation transport activity in MEM. However, these results should not be interpreted as a lack of unidirectional FS Na and K fluxes in VEC, but rather as the result of a balance between FS influx and efflux of Na and K leading to a FS *net* flux not different from zero.

The effect of medium substitution on OS net Na and K fluxes can be also seen for the cell line BFAE-39 which was incubated in another cultured medium, HF12, and in BSS. Table 3 reveals that the OS Na

Table 3. Effect of medium composition on ouabain-sensitive net Na and K fluxes in cells BFA-1c and BFAE-39

Cells	Medium	Ouabain-sensitive net flux	
		K	Na
		(nmol/mg protein · min)	
BFA-1c[a]	MEM[c]	−10.7 ± 1.4	6.2 ± 0.9
BFA-1c	BSS[d]	−6.0 ± 0.4*	2.4 ± 0.6*
BFAE-39[b]	HF12[c]	−13.8 ± 2.6	11.4 ± 1.7†
BFAE-39	BSS[d]	−4.7 ± 0.4*	2.6 ± 0.4‡

$\bar{X}$ ± SEM; $n = 4$; [a] *vs.* [b]: † $P < 0.05$; [c] *vs.* [d]: * $P < 0.01$, ‡ $P < 0.005$.

gain and K loss were significantly lower in BFAE-39 cells maintained in BSS as compared to HF12.

Based on these observations it was concluded that a component present in cultured medium was absent in BSS. To test this hypothesis the rates of OS net Na gain and K loss were measured in media of different compositions. Figure 2 reveals the typical reduction of the OS net cation movements in BFAE-39 cells kept in BSS (condition *B*) as compared to HF12 (condition *A*). Presence of phosphate (condition *C*), bicarbonate (condition *D*), or their combination (condition *E*) and with trace elements (condition *F*) in modified BSS (MBSS, *see* Materials and Methods) restored the rates of OS cation fluxes to the levels observed in culture medium. However, it should be noted that the presence of phosphate restored the OS K more than Na flux.

It is known that VEC possess a OR and FS Na–K–Cl cotransport [9, 18]. To test the hypothesis whether bicarbonate and phosphate were responsible for the effects observed on the OS cation fluxes (Fig. 2), the net Na and K fluxes were measured under the same conditions as Fig. 2 but in the presence of ouabain and furosemide (to inhibit the Na–K–Cl cotransport). Figure 3 shows that furosemide inhibited the OR Na flux by 50% in BSS-incubated cells only, and had no effect on transport in cells maintained in the culture medium or in the bicarbonate- or phosphate-substituted media. Addition of serum, vitamins, amino acids and lipids to BSS at the concentrations present in CM did not restore the OS and OFR Na and K fluxes to the levels observed in the control. Hence, it can be concluded that bicarbonate and phosphate in MBSS induced ouabain- and furosemide-resistant cation fluxes of similar magnitude to those determined in culture medium. Furthermore, as observed in Fig. 1, these results imply that in CM, a FS *net* component for Na and K appears to be negligible.

In Table 3 it was shown that the OS net Na and

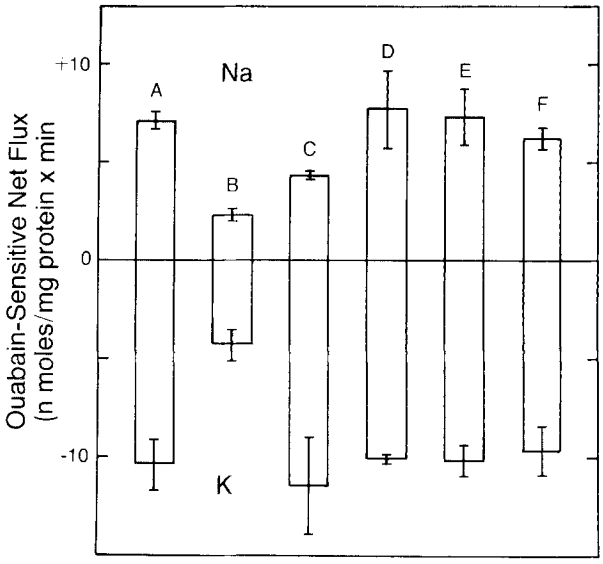

Fig. 2. Effect of medium composition on ouabain-sensitive net Na and K fluxes (Clone BFAE-39). Conditions: *A*, culture medium, HF-12, buffered with 14 mM bicarbonate, plus 1 mM phosphate; *B*, BSS buffered with 20 mM HEPES; *C*, similar to BSS but with 1.9 or 10 mM phosphate replacing 20 mM HEPES; *D*, as in condition *C*, 14 mM bicarbonate replaced 20 mM HEPES: *E*, this medium contained both, 1.9 mM phosphate and 14 mM bicarbonate; and *F*, as *E* but with the addition of trace elements at the concentrations in the culture medium (*see* Materials and Methods for further details). Cells were washed and equilibrated for 45 min in each medium but without inhibitors. The medium was aspirated, and fresh medium containing either none, 2 mM ouabain, or 2 mM ouabain plus 1 mM furosemide was added and incubated for 30 min longer. Data represent means ± SEM of 3–5 different experiments with triplicate determinations per point. Difference between mean values, conditions *A* and *C*, $t = 5.838$, degrees of freedom = 6, $P < 0.005$. Passages and days in culture were from P_6D_6 to $P_{10}D_5$

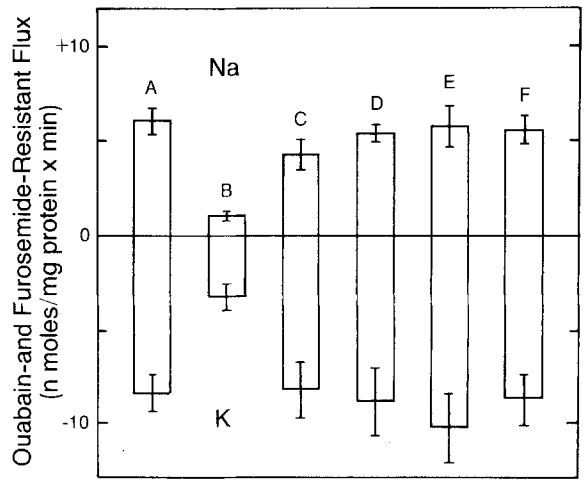

Fig. 3. Effect of medium composition on ouabain- and furosemide-resistant net Na and K fluxes (Clone BFAE-39). Conditions and experimental design were the same as in legend to Fig. 2

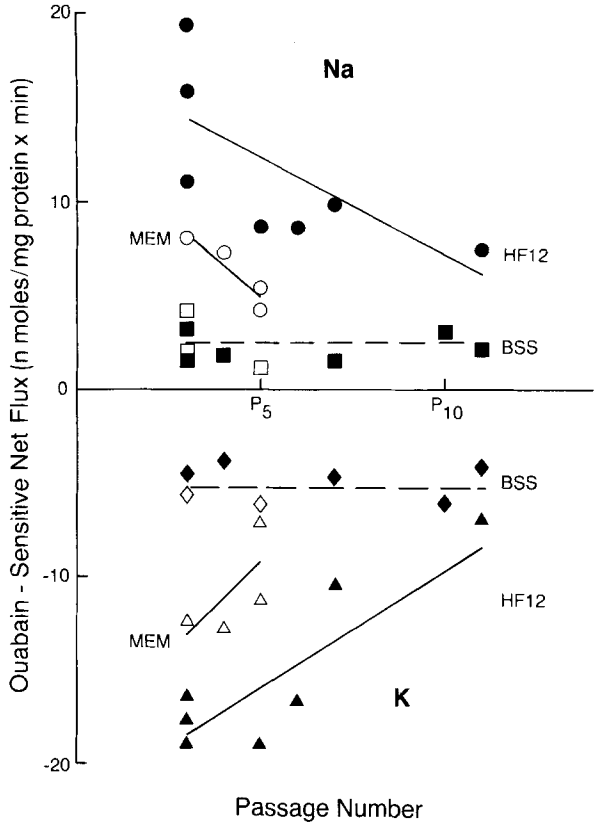

Fig. 4. Ouabain-sensitive net Na and K fluxes as a function of passage number. Clones BFA-1c (open symbols) and BFAE-39 (filled symbols). Cells assayed at confluence from days 4–8. Equilibration time was 10 min. Slopes were obtained by linear regression

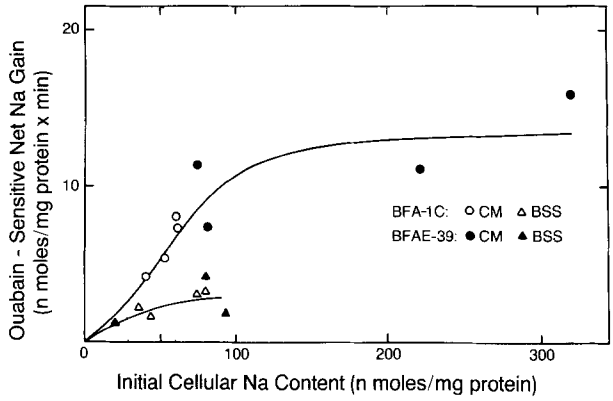

Fig. 5. Effect of initial cellular Na content on ouabain-sensitive net Na gain in CVEC. Cells from clone BFA-1c (○,△) and BFAE-39 (●,▲) were incubated in culture medium (○,●) or in BSS (△,▲), in the absence and presence of 2 mM ouabain. Equilibration time was 10 min. The solid lines were drawn by eye

K movements were 80 and 30%, respectively, higher in BFAE-39 compared to BFA-1c cells. This finding might be due to difference in cellular age, a hypothesis tested in the experiments of Fig. 4 which shows that in culture medium but not in BSS, there was an inverse correlation between OS Na and K fluxes and passage number for both cell types. The statistical parameters for a larger sample than that of Fig. 3 (line BFAE-39) were for Na: $r = 0.63$, $P < 0.01$, $n = 15$; and for K: $r = -0.79$, $P < 0.0005$, $n = 15$. In addition, and apparently correlated with a larger number of cumulative population doublings (CPD) of cells from clone BFA-1c (more than 70), the OS net Na and K fluxes in these cells was lower than in BFAE-39 cells (CPD about 30). The presence of fluorsemide did not change these relationships (*data not shown*), further suggesting that the larger Na and K movements in CM were dependent on bicarbonate and phosphate, although the precise mechanism is not clear at this point.

One possible explanation for the age-dependent decrease of OS net Na and K fluxes is that older cells contain less Na and K, resulting in a reduced cation gradient across the membrane and thus affecting those transport mechanisms dependent on these gradients. To support this proposition the OS cation fluxes were plotted as a function of the initial cellular Na and K content. Figure 5 shows that the rates of OS Na gain in cultured medium but not in BSS were higher for the younger BFAE-39 cells than for the older BFA-1c cells. A negative correlation between initial Na and passage number ($P < 0.01$) supports this observation. The figure also shows that the OS Na flux in the two cell types in culture medium was higher at any given Na content than for cells incubated in BSS as was already seen in Table 3. Similar results were obtained for OS net K fluxes (*not shown*).

Discussion

The decrease in OS and OFR net cation fluxes (Figs. 1–5, and Table 3) by incubation of VEC in BSS may be due to either removal of bicarbonate and phosphate or to addition of HEPES to BSS. The first possibility requires that VEC possess OFR pathways for Na and K which are bicarbonate- and phosphate-dependent. These pathways have been identified in corneal and capillary endothelial cells and in cells from other tissues. For example, an electrogenic Na–HCO_3 cotransport exists in the apical membrane of corneal EC [15, 25], and preliminary studies with fluorescent probes point to the presence of such pathways in capillary EC from adrenal medulla [23]. In addition, a Na-phosphate cotransport is present at the luminal surface of proximal tubules

[26] and the small intestine [10]. Alternatively, HEPES may have induced changes in intracellular pH [7, 8] or may have caused a direct effect on one or more ionic pathways. Because MEM also contained HEPES (25 mM) in addition to bicarbonate (26 mM), and the effect of buffer substitution was observed in BFA-1c cells (Figs. 1 and 3–5, and Table 3) regardless of the presence of this buffer, the present results seem to support the existence of prominent bicarbonate- and phosphate-dependent Na and K leak pathways in CVEC. The present results also emphasize the importance of an adequate selection of buffer substitution to study ion transport in cultured vascular endothelial cells.

Substitution of Na and bicarbonate in media bathing corneal endothelial cells induces alterations in intracellular pH and under certain conditions in membrane potential [6, 14]. Thus, it is likely that these parameters were altered upon medium substitution in vascular endothelial cells. However, in the studies on corneal endothelial cells the changes of media were produced in a relatively short time, whereas in the present study the cells were equilibrated in each medium prior to the measurement of cation fluxes. Further studies are necessary to determine the effect of medium substitution on membrane potential and intracellular pH in vascular endothelial cells.

The OS net Na gain in cells incubated in phosphate alone was lower than in CM (Fig. 2). This effect of phosphate cannot be explained by lack of buffer capacity at 1.9 mM phosphate because it also appeared at 10 mM phosphate. One possibility is that phosphate may affect an OR pathway that transports Na but not K because the net K loss was the same as that in culture medium. This finding deserves further study.

Endothelial cells are recognized as a good model to study the aging process [13]. In the present report, OS and OFR net Na and K fluxes decreased with increasing passage number in CM but not in BSS (Fig. 4), where the bicarbonate- and phosphate-dependent components seem to be present. A possible explanation for these findings is that the changes in OS and OFR fluxes were due to cell senescence, since the cells used in this study are known to age in culture [17, 22]. Therefore, these results suggest that bicarbonate- and phosphate-dependent Na and K pathways may play a role in cellular aging. Although some recent studies have reported a decrease in ion transport (mainly Na/K pump and Na, K ATPase activities), with age in vivo or in vitro [1, 5, 16, 24, 27], this is the first study of that kind in cultured vascular endothelial cells. Thus, the study of ion transport systems in cultured vascular endothelial cells may serve as a biological marker to understand the aging process in vitro, and also in vivo.

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