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K^+ Transport in 'Tight' Epithelial Monolayers of MDCK Cells

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Summary. Bidirectional transepithelial K^+ flux measurements across 'high-resistance' epithelial monolayers of MDCK cells grown upon millipore filters show no significant net K^+ flux.

Measurements of influx and efflux across the basal-lateral and apical cell membranes demonstrate that the apical membranes are effectively impermeable to K^+.

K^+ influx across the basal-lateral cell membranes consists of an ouabain-sensitive component, an ouabain-insensitive component, an ouabain-insensitive but furosemide-sensitive component, and an ouabain- and furosemide-insensitive component.

The action of furosemide upon K^+ influx is independent of $(Na^+ - K^+)$-pump inhibition. The furosemide-sensitive component is markedly dependent upon the medium K^+, Na^+ and Cl^- content. Acetate and nitrate are ineffective substitutes for Cl^-, whereas Br^- is partially effective. Partial Cl^- replacement by NO_3 gives a roughly linear increase in the furosemide-sensitive component. Na^+ replacement by choline abolishes the furosemide-sensitive component, whereas Li^+ is a partially effective replacement. Partial Na^+ replacement with choline gives an apparent affinity of ~7 mM Na, whereas variation of the external K^+ content gives an affinity of the furosemide-sensitive component of 1.0 mM.

Furosemide inhibition is of high affinity ($K_{\frac{1}{2}}$=3 μM). Piretanide, ethacrynic acid, and phloretin inhibit the same component of passive K^+ influx as furosemide; amiloride, 4,-aminopyridine, and 2,4,6,-triaminopyrimidine partially so. SITS was ineffective.

Externally applied furosemide and Cl^- replacement by NO_3^- inhibit K^+ efflux across the basal-lateral membranes indicating that the furosemide-sensitive component consists primarily of K:K exchange.

Key words K^+ fluxes · MDCK · ouabain · furosemide · cultured epithelium · $Na^+ + K^+ + Cl^-$ cotransport

Introduction

A knowledge of the K^+ transport mechanisms in epithelia is important, both for an understanding of net transepithelial K^+ ion transport, e.g., by renal tubular epithelium (Giebisch, 1979; Giebisch & Stanton, 1979) and for elucidating the relationship between cellular ion exchanges (in which normal cellular cation gradients are maintained) and transcellular ion transports, principally of Na^+ (Nellans & Schultz, 1976). In addition, intracellular K^+ has been implicated in a variety of cellular control mechanisms and Civan (1980) has suggested a direct regulation of transcellular Na^+ transport by intracellular levels of K^+ activity.

Despite the obvious importance of K^+ transport by epithelial tissues, little is known about the kinetics of K^+ exchanges and the processes responsible for such exchanges. Considerable information regarding Na^+ transport mechanisms in epithelia has been previously derived from the use of appropriate model systems such as anuran skins and bladders. We have chosen to investigate some aspects of transepithelial and transcellular K^+ exchanges using a model tissue culture epithelium, MDCK, which forms epithelia consisting of a single monolayer of cells of high transepithelial electrical resistance, some of whose ion transport characteristics have been described (Simmons, 1981*a*). Our major finding is the identification of a component of the K^+ influx across the basal cell membranes of MDCK cells which is insensitive to ouabain but is markedly inhibited by furosemide and is dependent upon the medium Na^+ and Cl^- contents. Some of the present results have appeared in abstract form (Aiton et al., 1981).

Materials and Methods

Cell Culture

Experiments were performed upon MDCK dog kidney cells of 60–72 serial passages obtained from Flow Laboratories (Irvine, Scotland). The physiological and biochemical properties of these cells and epithelial monolayers have been described (Barker & Simmons 1981; Richardson, Scalera & Simmons 1981; Simmons, 1981*a*) and differ substantially from literature values for MDCK cells and epithelial monolayers of 100 serial passages (Cereijido et al. 1978; Barker & Simmons, 1981; Richardson et al., 1981). The cells were maintained in serial culture in Minimum Essential Medium Eagles (M.E.M.E.) with 10% vol/vol fetal bovine serum, gentamycin antibiotic, and amino acid supplements at 37 °C in 95% air/5% CO_2 atmosphere, as previously described (Simmons, 1981*a*). Subculture and preparation of epithelial monolayers upon

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millipore filters by seeding at high density was identical to that previously described (Simmons, 1981*a*). In addition, monolayers were grown upon plastic petri-dishes (Sterilin, 5 cm radius) after seeding at 0.25×10^6 cells/plate in 5 cm^3 of growth medium for influx determinations. For experimental purposes (3–4 days of growth), the cell monolayers were still sub-confluent, thus allowing access of solution to the basal aspects of the cell layer. The final cell density was 2 to 3×10^6 cells per petri dish.

Electrical Measurements

Cell monolayers were mounted in Ussing chambers (0.75 cm window radius, 1.76 cm^2 exposed monolayer), thermostated at 37 °C for measurement of PD and resistance in a similar fashion to rabbit small intestine (Simmons & Naftalin, 1976; Naftalin & Simmons, 1979). An automatic voltage clamp (Simmons & Naftalin, 1976) was connected to the Ussing chamber via matched calomel half cells (for potential measurement), silver/silver chloride half cells (for current passage), and saturated KCl-agar salt bridges. Resistance determinations were made routinely by passing 2 μA hyper-polarizing current pulses across the cell monolayer.

Transepithelial K^+ Flux Measurements

Bidirectional K^+ fluxes were determined simultaneously on the same cell monolayer under voltage clamp (PD = 0 mV) using ^{42}K and ^{86}Rb (Radiochemical Centre, Amersham, U.K.) as tracers of the bidirectional K flux. Solutions were not gassed because of the inclusion of 1% vol/vol fetal bovine serum in the experimental media. The flux period was for 1 hr following an initial 1 hr equilibration. Total chamber volumes were 14 cm^3 and sample volume 1 cm^3. Isotope equilibration was never greater than 1%. ^{42}K was used to measure the unidirectional flux from apical to basal solutions (J_{a-b}) and ^{86}Rb was used to trace the reverse flux (J_{b-a}). The use of ^{86}Rb as an adequate transepithelial tracer of K flux was checked in separate experiments where the simultaneous determination of bidirectional transepithelial K^+ fluxes was made using ^{42}K together with ^{86}Rb (Fig. 1). Over a wide range of fluxes, ^{86}Rb can be seen to be an adequate tracer of K^+ flux.

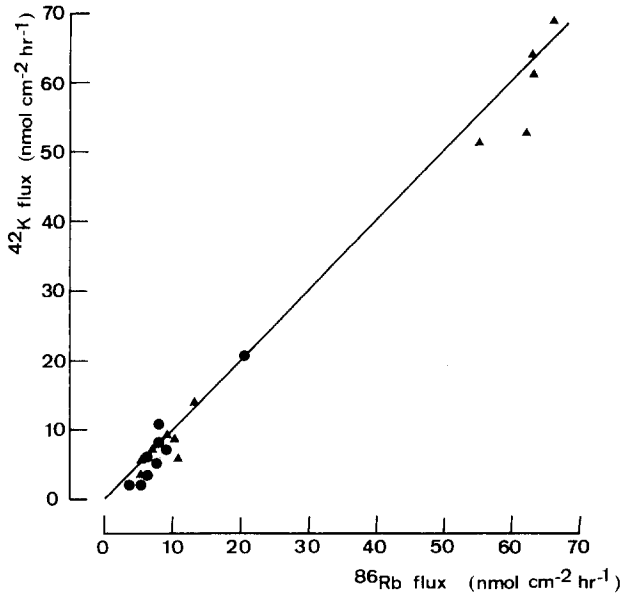

Fig. 1. Validation of the use of ^{86}Rb as an adequate tracer of the bidirectional transmonolayer K^+ fluxes. ^{86}Rb and ^{42}K were used to simultaneously measure a unidirectional flux (*see* Methods). (●) Apical to basal $^{42}K/^{86}Rb$ influx, (▲) basal to apical $^{42}K/^{86}Rb$ flux. The solid line represents a 1:1 relationship. Regression analysis of the experimental data give a slope of 1.00 ± 0.03 (SE) and a correlation coefficient (r) of 0.99 ($n = 22$, $P < 0.001$)

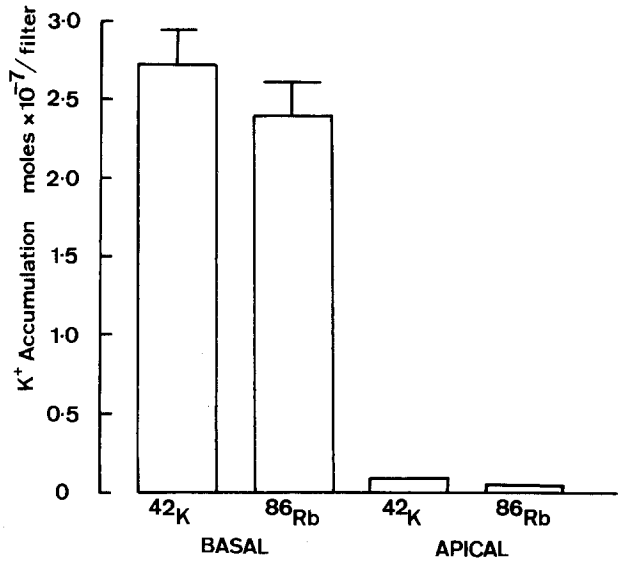

Fig. 2. Intracellular K^+ accumulations within the cells of epithelial monolayers determined using ^{42}K or ^{86}Rb simultaneously from either the apical or basal bathing solutions. Total equilibration was for 2 hr

Table 1. Time course of equilibration of cellular ^{86}Rb (cells grown upon petri dishes)

Time (hr)	n	^{86}Rb concentration $t = x$ / ^{86}Rb concentration $t = 3$ hr
0.5	3	0.31 ± 0.10
1.0	3	0.56 ± 0.08
2.0	3	0.92 ± 0.09
3.0	3	1.0

In addition to transepithelial K^+ fluxes, the tissue K^+ content was determined after washing (4×) in ice-cold choline Cl solution (total wash time < 1 min) and the ratio of ^{42}K to ^{86}Rb within the intracellular pool determined (Simmons & Naftalin, 1976; Naftalin & Simmons, 1979). The relative accumulations of ^{42}K and ^{86}Rb across either apical or basal cell borders after 2-hr incubations (determined simultaneously), is shown in Fig. 2 and shows that ^{86}Rb is an adequate measure of K^+ accumulations. Table 1 shows the time course of equilibration of cellular ^{86}Rb with cells grown upon petri dishes. Equilibration is observed by two hours. The calculated K^+ content using ^{86}Rb distributions after a 3-hr incubation gave a K^+ concentration of 177 ± 5 mM ($n = 10$). This compares with 192 ± 10 mM ($n = 10$) for K^+ contents measured by flame photometry using the same cell batch; this suggests that the greater proportion (92%) of cell K^+ is exchangeable with radioisotope.

^{42}K activity was measured in mixed samples of ^{42}K and ^{86}Rb using a Packard Autogamma counter. Correction of ^{42}K activity for ^{86}Rb activity was made by recounting all samples following ^{42}K decay (12 half lives). ^{86}Rb activity was counted after ^{42}K

decay by its β-emissions in a Packard liquid scintillation spectrometer (model 3255) in scintillation cocktail (toluene, Triton X-100 45/45 vol/vol, and Scintol 10 vol/100 vol).

Measurements of K^+ Influx Across the Basal and Apical Aspects

Cell monolayers were mounted in Ussing type-chambers (1.76 cm^2 of exposed monolayer), allowing access to the apical/basal solutions. Influx measurements were initiated by adding ^{86}Rb to the appropriate bathing solution. Solutions were mixed by small stir bars. After the appropriate time, the radioactive solution was removed by suction, the monolayer was washed (4×) by ice-cold choline Cl (1 min), removed from the Ussing chamber, and blotted on Whatman No. 1 filter paper. Isotope was then extracted in distilled water for three hours.

Influx measurements are expressed relative to cell water, estimated by releasing cells from several epithelial monolayers of the same batch by trypsin-EDTA treatment and counting cell number/volumes by electronic sizing using a Coulter Counter/Channelyser (*see* below).

Measurements of K^+ Efflux across the Apical and Basal-Lateral Cell Surfaces

Cell monolayers were preloaded for 5 hr in ^{86}Rb Krebs clamped into Ussing-type chambers (1.76 cm^2 exposed monolayer) at 37 °C and the apical and basal effluxes measured simultaneously by successive addition and collection of 5 cm^3 aliquots of Krebs from the apical and basal ports at 2-min time intervals. At the end of the experiment, cell monolayers were extracted for the remaining ^{86}Rb content. Since both apical and basal effluxes were measured on a single monolayer, apical fractional efflux values were calculated using the known decrease in intracellular ^{86}Rb content due to loss of isotope across both the apical and the basal aspects of the cell layer.

Measurements of K^+ Influx in Sub-Confluent Cell Monolayers Grown upon Petri Dishes

^{86}Rb was used as an isotopic tracer to indicate potassium movements. This was checked for a range of K^+ influxes by simultaneous measurement of K^+ influx using ^{42}K and ^{86}Rb (Fig. 3). Potassium influxes measured using ^{86}Rb do not deviate significantly from a 1:1 ratio with ^{42}K measured influxes. Potassium influxes were measured during a 5-min incubation at 37 °C in a Krebs' solution containing 0.2 $\mu Ci/cm^3$ ^{86}Rb, to ensure that all flux measurements were made in the linear portion of the uptake curve (*see* Fig. 4). At the end of the influx period, the cell layer was rinsed 4× with ice-cold Krebs' (~20 sec) to remove extracellular isotope and then treated with trypsin (0.1% Flow Laboratories)-EDTA (0.1 mM) in Ca−Mg free Earle's saline (Flow Laboratories) for ~15 min to form a single cell suspension. The washing protocol was checked by loss of ^{14}C-inulin in separate experiments under identical conditions. After trypsinization, the cell number and cell volume of an aliquot of each experimental plate was determined on a Coulter Counter (ZF) with Channelyser (C1000) (Boardman et al., 1974; Aiton & Lamb, 1980). This method of preparing a cell suspension does not affect cell volume compared to substrate-attached cells (Burrows & Lamb, 1962; Simmons, 1981*b*). The ^{86}Rb content of samples were measured in the Packard liquid scintillation spectrometer by the Cerenkov effect. Mixed samples of ^{42}K and ^{86}Rb were counted as described above.

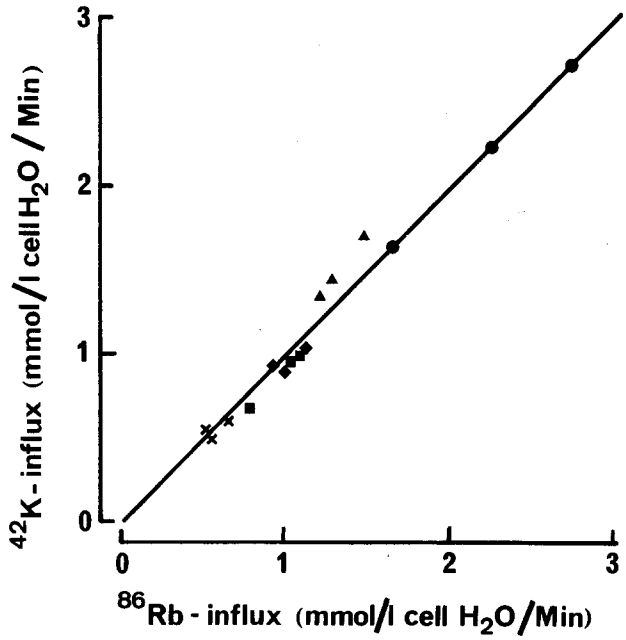

Fig. 3. Validation of the use of tracer ^{86}Rb influx as a measure of K^+ influx on cells grown upon petri dishes. Simultaneous measurements of ^{42}K and ^{86}Rb tracer influx were made as described in Methods. The solid line represents a 1:1 relationship. Regression analysis of the experimental data give a slope of 0.95±0.05 (SE) $n=15$, and a regression coefficient (r) of 0.98 ($P<0.001$). Total flux (●) 1×10^{-3} M ouabain (■), $+1\times10^{-4}$ M furosemide (▲), $+1\times10^{-3}$ M ouabain and 1×10^{-4} M furosemide (×)

K^+ Efflux from Sub-Confluent Cell Monolayers Grown upon Plastic Petri Dishes

Successive 5 cm^3 aliquots of Krebs' were added to, and collected from, plates of cells preloaded with ^{86}Rb (Aiton & Lamb, 1980). The ^{86}Rb content of the cells was estimated at the end of the efflux period in aliquots prepared by trypsin treatment (*see* above).

Estimation of Intracellular Cation Contents

After incubation in the appropriate experimental solution for various times the subconfluent cell layers were washed in ice-cold isotonic choline chloride as previously described. The cell layer was then extracted in 3 cm^3 of double distilled water and the Na and K measured by flame photometry. The cell number and volume were determined on separate cell layers as described above.

Experimental Solutions

Experiments were carried out, except where otherwise stated, in a Krebs' solution containing (in mM): 137, NaCl; 5.4, KCl; 2.8, $CaCl_2$; 1.2, $MgSO_4$; 0.7, H_2O; 0.3, NaH_2PO_4; 0.3, KH_2PO_4; 12, HCl; 14, Tris base; 11, glucose supplemented with 1.0% vol/vol fetal bovine serum, pH 7.4. In some later experiments the fetal serum was substituted for calf new-born serum, or horse donor serum.

The Na^+ content of the experimental media was varied by isosmotic replacement of NaCl by choline Cl and LiCl. The Cl^- content was varied by isosmotic substitution of NaCl by NaBr, NaI, $NaNO_3$, and Na acetate. In some cases Tris maleate replaced

Tris Cl. The K^+ content was varied from 0 to 9 mM KCl without adjustment of the osmolarity.

For experiments where the Na^+, Cl^- or K^+ content of the media was varied, serum was dialyzed overnight against two changes of $\times 50$ volumes distilled water.

Materials

Where possible, inorganic salts of Analar quality were used.

The stilbene, 4-acetamido-4′-iso-thiocyanato-stilbene-2,2′-stilbene disulphonic acid (SITS) was obtained from B.D.H. Chemicals (Poole, U.K.) and dissolved in 10 mM Tris base to make a stock solution.

4,-aminopyridine, 2,4,6,-triaminopyrimidine, phloretin, and ouabain were obtained from the Sigma Chemical Co. (Poole, U.K.); stock solutions were made in Krebs' (4,-aminopyridine, 2,4,6,-triaminopyrimidine) 10 mM Tris and distilled water, respectively.

Ethacrynic acid and amiloride were gifts from Merck, Sharp and Dohme; stock solutions were made in 10^{-2} M Tris and Krebs', respectively.

Furosemide and piretanide were gifts from Dr. S. Dombey of Hoechst (Hounslow, Middlesex, U.K.). Stock solutions were made in 10^{-2} M Tris.

Statistics

Variation in results is expressed as the standard error of the mean (SEM). Tests for significance of differences were made by a two-tailed Student's *t* test (unpaired mean's solution). Linear regression analysis was performed as described by Snedecor and Cochran (1968) on an Olivetti P6060 minicomputer.

Results

Transepithelial K^+ Fluxes

Epithelial monolayers of MDCK cells of between 60 and 66 serial passages were previously shown (Simmons, 1981*a*) to maintain only a small short-circuit current (0.5 μA cm^{-2}) and display a high transepithelial resistance (4.0 kΩ cm^2). Table 2 shows that in the present series of experiments, an identical pattern of high electrical resistance and low short-circuit current was observed with epithelial monolayers prepared over a slightly wider range of serial passages (up to 72 passages).

Measurement of the bidirectional K^+ fluxes using ^{42}K and ^{86}Rb indicate that there is no significant net K^+ flux in short-circuit conditions ($P > 0.1$) (Table 2). The bidirectional transepithelial K^+ permeabilities ($P_{a-b} = J_{a-b}/C_a$ where C_a is the concentration of ion in compartment *a*) are 0.70 and 0.88 cm^{-1} $hr^{-1} \times 10^{-3}$ for P_{a-b} and P_{b-a}, respectively, similar in magnitude to the bidirectional transepithelial permeabilities for Na^+ and Cl^- (Simmons, 1981*a*).

Since the $(Na^+ - K^+)$-ATPase is preferentially located upon the lateral cell membranes in MDCK cells (Lamb, Ogden & Simmons, 1981; Simmons, 1981*a*), the present observation of an absence of net K^+ secretion implies that the apical cell membrane is effectively impermeable to K^+ movement. Table 2 shows that the ratio of isotope ($^{86}Rb/^{42}K$) within the tissue at the end of the flux measurement period is 19.40, consistent with a low permeability of the apical cell membrane with respect to the active and passive K^+ permeabilities of the basal-lateral membranes.

K^+ Influx and Efflux across Apical/Basal-Lateral Aspects

Figure 4 shows the time dependence of influx across the apical and basal-lateral membranes of intact epithelial layers of MDCK cells. K^+ influx across the basal-lateral membranes is some $100\times$ greater than that across the apical membrane. The magnitude of the apical K^+ influx is only 0.003 μmol cm^{-2} hr^{-1}, suggesting that the greater proportion of the bidirectional transepithelial K^+ fluxes (Table 2) are mediated by a paracellular shunt pathway which may result from edge damage (Simmons, 1981*a*). The comparable magnitudes of the Na^+, K^+ and Cl^- bidirectional transepithelial permeabilities supports this view (*see* above).

Figure 5 shows the fractional loss of K^+ across the apical and basal-lateral cell aspects. Although the fractional loss across the basal cell aspects is time dependent to 12 min indicating diffusion restriction in the unstirred layers of the millipore filter, the basic pattern of the relative impermeability of the apical membrane to K^+ is evident. That K^+ loss across the basal aspects is only 25-fold greater than apical loss indicates that some loss of K^+ occurs across the epithelium via paracellular shunt pathways from the basal to the apical solution. 1×10^{-3} M ouabain

Table 2. Transmonolayer bidirectional K^+ fluxes determined using ^{42}K and ^{86}Rb for apical to basal K flux (J^K_{a-b}), respectively[a]

K ion concentration (mmol/liter)	*n*	J_{a-b} μmol (cm^{-2} hr^{-1})	J_{b-a} μmol (cm^{-2} hr^{-1})	J_{net} μmol (cm^{-2} hr^{-2})	Short-circuit current equivalent (μmol cm^{-2} hr^{-1})	Conductance (mho cm^{-2} $\times 10^3$)	Tissue isotope ratio ($^{86}Rb/^{42}K$)	Tissue accumulation (moles $\times$ 10^{-7}/filter)
5.4	22	0.038 ± 0.014	0.048 ± 0.014	−0.010 ± 0.009	0.033 ± 0.005	0.45 ± 0.07	19.40 ± 3.56	2.82 ± 0.18

[a] Epithelial monolayers were voltage-clamped to zero PD, and flux measurements were performed over 1-hr periods. All errors are ± SE.

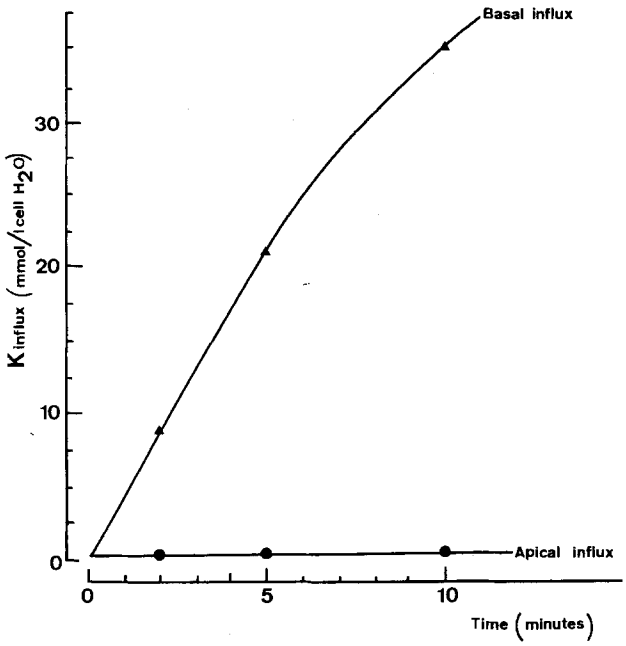

Fig. 4. Time course of K^+ influx (using ^{86}Rb as tracer) across the basal and apical aspects of epithelial MDCK monolayers grown on Millipore filter and clamped in Ussing-type chambers. Each point is the mean of two observations

reduces the magnitude of basal K^+ influx (Table 3) consistent with the autoradiographic (Lamb et al., 1981) and physiological (Simmons, 1981*a*) localization of the (Na^+-K^+)-ATPase. In the presence of ouabain there remains a substantive portion of the K^+ influx which is further reduced by furosemide (1×10^{-4} M). Neither ouabain nor furosemide affect the influx of K^+ across the apical cell membrane, which in all three conditions remains a small (1%) fraction of the total cellular K^+ influx. Measurements of total K^+ influx (and efflux) upon subconfluent MDCK cells grown upon plastic petri dishes will, therefore, reflect K^+ transport across the basal-lateral aspects of the MDCK cell. For reasons of convenience and cost, most of the present measurements were made on MDCK cells grown on plastic petri dishes.

Additive Effects of Ouabain and of Furosemide upon K^+ Influx

Figure 6 shows the effect of furosemide and ouabain upon K^+ influx into MDCK cells grown to subconfluent density on plastic petri-dishes. The magnitude of the control K^+ influx and its sensitivity to ouabain and furosemide is comparable to that obtained for total basal influx in intact monolayers (Table 3). This indicates that there is no diffusion restriction to K^+ across the basal surfaces and that subconfluent cells

Table 3. K^+ influx into epithelial monolayers of MDCK cells clamped in Ussing-type chambers under voltage clamp[a]

	K influx (mmol/liter cell water/min)	
	Apical influx	Basal influx
Control	0.02±0.01 (3)	2.58±0.21 (11)
$+1\times10^{-3}$ M ouabain	0.04±0.01 (3)	1.82±0.14 (11)
$+1\times10^{-3}$ M ouabain $+1\times10^{-4}$ M furosemide	0.03±0.01 (3)	1.08±0.20 (11)

[a] Cell number/volume measurements were made by Coulter Counter by releasing cells with trypsin-EDTA in a parallel experiment. Results are expressed as the mean ±SE. The number of separate determinations are in parentheses.

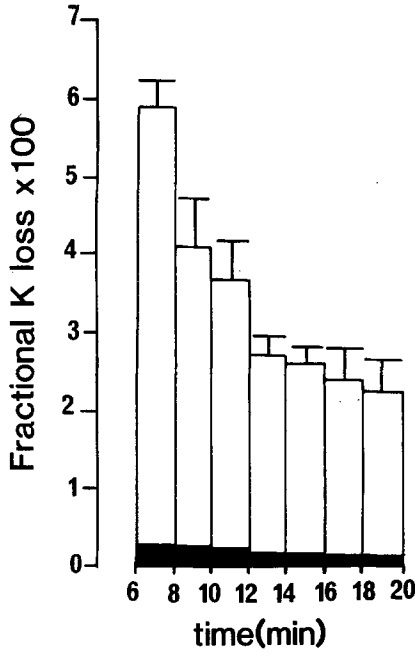

Fig. 5. Efflux of K^+ across the apical and basal aspects of intact MDCK epithelial monolayers mounted in Ussing chambers made by successive addition and collection (2 min) of 5-cm^3 aliquots of Krebs. The first three washings were discarded. Each observation is the mean±SE of three determinations. The mean monolayer resistance was 1.75±0.33 (SE) $k\Omega cm^2$. Open columns, efflux from the basal surfaces; filled columns, efflux from the apical surfaces.

display properties similar to intact epithelial monolayers. At considerably lower cell densities than those presently used (0.2×10^6 to 1×10^6 cell per plate) an elevated total K^+ influx is observed; however, the quality of the ouabain-sensitive and furosemide-sensitive fluxes is not altered (*unpublished observations*). Figure 6 shows that the effects of ouabain (1×10^{-3} M) and furosemide (1×10^{-4} M) are approximately additive, suggesting independent actions. The ouabain-sensitive component of K^+ influx is 1.19±

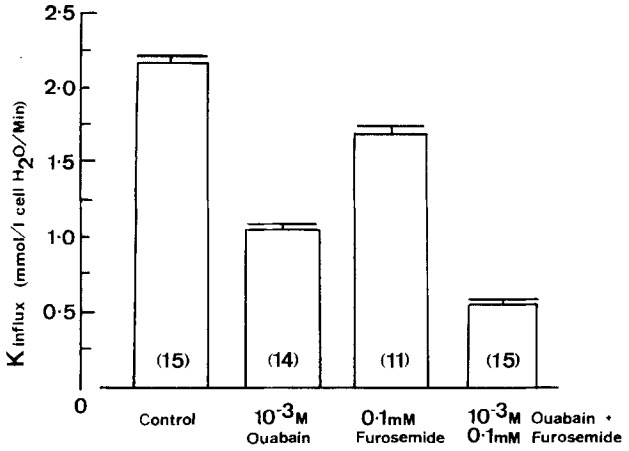

Fig. 6. Additive effects of ouabain (10^{-3} M) and furosemide (10^{-4} M) upon the K^+ influx. The numbers in parentheses are the number of individual determinations, pooled results from four separate experiments. Error bars are ±SE

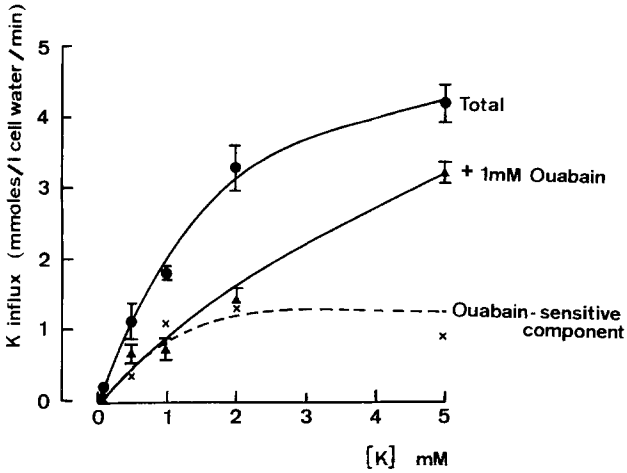

Fig. 7. Effect of variation in medium K^+ content upon K^+ influx in the presence (▲) and absence (●) of 1×10^{-3} M ouabain. The petri dishes were preincubated in the appropriate experimental solution for 10 min before initiation of the influx. Each point is the mean ±SE of three determinations. (×) Ouabain-sensitive component of K^+ influx. Solid lines in this and subsequent figures were fitted by eye

Table 4. The effect of furosemide upon intracellular cation concentrations[a]

Condition	Incubation time (hr)	Na^+ (mmol/liter cell water)	K^+ (mmol/liter cell water)
Control	1	26±3(3)	164±3(3)
	2	27±3(3)	172±5(3)
0.1 mM furosemide	1	35±1[b](3)	173±4(3)
	2	36±1[b](3)	189±3[b](3)
0.1 mM ouabain	1	86±7[c](3)	71±2[c](3)
	2	96±8[c](3)	91±1[c](3)

[a] Incubations were performed in the standard Krebs solution at 37 °C. The number of separate determinations are in parentheses. Significantly different from control values: [b] $P<0.05$; [c] $P<0.01$.

0.06 and 0.95±0.06 mmol liter^{-1} cell water min^{-1} in the absence and presence of furosemide, respectively, whereas the furosemide-sensitive component of K^+ influx is 0.73±0.07 and 0.50±0.06 mmol liter^{-1} cell water min^{-1} in the absence and presence of ouabain. The absence of significant effects of furosemide upon (Na^+-K^+)-pump function is supported by measurements of intracellular ion concentrations (Table 4) which show that intracellular K^+ levels are maintained despite a 2-hr incubation with furosemide. In contrast, the presence of 0.1 mM ouabain causes a marked reduction in intracellular K^+ and rise in intracellular Na^+.

A small, though significant, increase in intracellular Na^+ concentration is observed with furosemide (Table 4) together with a small increase in intracellular K^+. The lack of effect of furosemide upon the (Na^+-K^+)-ATPase at 10^{-4} M parallels its lack of effect in isolated (Na^+-K^+)-ATPase preparations (Jorgensen, 1980).

K^+ influx across the basal membranes therefore comprises a ouabain-sensitive component, a furosemide-sensitive component, and finally a ouabain and furosemide-insensitive component; this pattern of K^+ fluxes is analogous to that observed in human red cells (Chipperfield, 1980, 1981; Dunham, Stewart & Ellory, 1980), ascites tumor cells (Bakker-Grunwald, 1978), and turkey erythrocytes (Bakker-Grunwald, 1981).

The Ouabain-Sensitive Component of the K^+ Influx

The (Na^+-K^+)-pump mediated K^+ influx displays similar characteristics with respect to its dependence upon medium K^+ (Fig. 7) and inhibition by ouabain (Lamb et al., 1981) as do other tissues, notably renal tubules (Jorgensen, 1980). The K_m for K^+ activation of the K^+ influx is 0.5 mM, and the K_i for ouabain inhibition (with a pre-exposure in K^+-free media) is $1-2 \times 10^{-7}$ M (Lamb et al., 1981) in good agreement with the K_m for ^{3}H-ouabain binding to these cells (Lamb et al., 1981). The substantive portion of the ouabain-insensitive K^+ influx has already been noted (Fig. 7); Fig. 7 also shows that the ouabain-insensitive K^+ influx is also nonlinearly related to medium K^+ concentration.

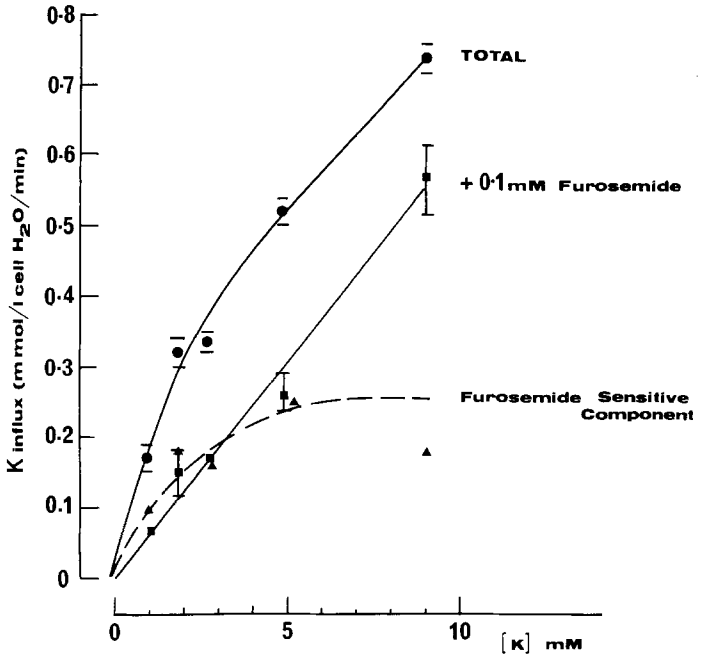

Fig. 8. Effect of variation in medium K^+ content upon the ouabain-insensitive K^+ influx (^{86}Rb as tracer) in the presence (■) and absence (●) of 0.1 mM furosemide. The furosemide-sensitive component (▲) is the difference between the total and furosemide-insensitive K^+ influxes. The petri dishes were preincubated in the appropriate experimental solution for 10 min before initiation of the influx. Each point is the mean ±SE of three determinations

The Furosemide-Sensitive Component of the K^+ Influx

By analogy to the behavior of the diuretic-sensitive, ouabain-insensitive K^+ fluxes in other cells (Wiley & Cooper, 1974; Bakker-Grunwald, 1978, 1981; Geck, Heinz, Pietrzyk & Pfeiffer, 1978; Chipperfield, 1980, 1981) we have examined the cation and anion dependence of the furosemide-sensitive K^+ influx.

K^+ Dependence. The K^+ concentration dependence of the furosemide-sensitive K^+ influx was determined in ouabain-containing media (Fig. 8). The furosemide-sensitive K^+ influx is dependent upon medium K^+ in a saturable fashion with a K_m of 1.0 mM. The ouabain-insensitive, furosemide-insensitive K^+ influx is, by contrast, linearly related to medium K^+ in the range to 0 to 10 mM K^+.

Na^+ Dependence. Replacement of medium Na^+ by either choline$^+$ or Li^+ reduces the furosemide-sensitive component of K^+ influx (Table 5); Li^+ is, however, a partial substitute for Na^+, and a significant furosemide component is observed. Complete replacement of medium Na^+ by choline$^+$ results in an increased ouabain and furosemide-insensitive K^+ influx (Table 5), indicating nonspecific actions of choline replacement.

The effect of partial replacement of Na^+ by choline$^+$ in the range 2.5 to 130 mM NaCl on the ouabain-insensitive K^+ influx is shown in Fig. 9. The furosemide-insensitive, ouabain-insensitive K^+ influx is largely independent of medium Na^+, whereas the furosemide-sensitive component is increased as medium Na^+ is raised in a saturable fashion. The K_m is 7 mM Na^+.

Table 5. Effect of Na^+ replacement upon ouabain-insensitive fluxes[a]

Ion replacement	K^+ influx (mmol/liter cell water/min)		
	Control	0.1 mM furosemide	Furosemide-sensitive component
NaCl	0.81 ± 0.05	0.30 ± 0.03	0.51 ± 0.06
Choline Cl	0.51 ± 0.06[c]	0.52 ± 0.04[b]	−0.01 ± 0.06[c]
LiCl	0.45 ± 0.03[c]	0.29 ± 0.04	0.16 ± 0.05[c]

[a] All determinations were made in the presence of 0.1 mM ouabain. Each determination is the mean ±SE of at least three separate determinations. A preincubation of 10 min in the appropriate experimental media was made prior to the K^+ influx measurent. Significantly different from NaCl values: [b] $P<0.05$; [c] $P<0.01$.

Anion Dependence. With complete substitution of the medium NaCl by bromide$^-$ acetate$^-$ or NO_3^- there was a reduction in the furosemide-sensitive K^+ influx component (Table 6) whereas acetate$^-$ and nitrate$^-$ were totally ineffective, Br^- was partially effective as a substitute for Cl^- (Table 6). Inclusion of 20 mM Na acetate to the normal Krebs also abolished the furosemide-sensitive K^+ influx component, indicating that, besides being an ineffective substituent anion, acetate$^-$ in the presence of Cl^- is an effective inhibi-

tor of the furosemide-sensitive K^+ influx (Table 6). Both NaBr and $NaNO_3$ are without inhibitory effect upon the furosemide-sensitive K^+ influx in the presence of NaCl (Table 6). Since Br^- is a partially effective substituent for Cl^- (*see* above), the chloride dependence of the furosemide-sensitive K^+ influx was determined using $NaNO_3$ with partial replacements (Fig. 10).

The ouabain and furosemide-insensitive K^+ influx is largely independent of medium Cl^- concentration, in the range 30 to 160 mM Cl^-. Occasionally an increase was observed with complete replacement as in Fig. 10, but this was not a consistent feature (*see* Table 6). The furosemide-sensitive K^+ influx displays a nonsaturating upward-curving dependence upon medium Cl^- concentration (Fig. 10).

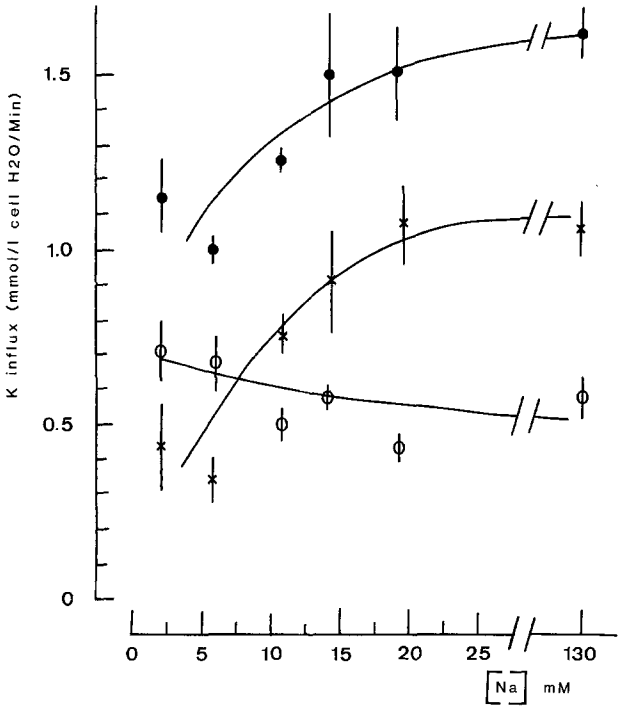

Fig. 9. Effect of medium Na upon the ouabain-insensitive K^+ influx NaCl was replaced isosmotically by choline Cl in the presence (○) and absence of (●) 1×10^{-4} M furosemide. The furosemide-sensitive component (×) is the difference between the ouabain-insensitive flux ± furosemide. Preincubation conditions were identical to those of Fig. 8. Each point is the mean ± SE of three determinations

Dose-Dependency of Furosemide Action

Figure 11 shows that the action of furosemide upon the ouabain-insensitive K^+ influx is of high affinity. Half-maximal inhibition is observed at 2–3 μM furosemide.

Table 6. Effect of anion replacement and anion inclusion upon the total ouabain-insensitive K^+ influx and the furosemide-sensitive K^+ influx component[a]

Na (anion)	K^+ influx (mmol/liter cell water/min)			
	Substitution of NaCl		Inclusion of 20 mM Na (anion)	
	Ouabain-insensitive	Furosemide-sensitive component	Ouabain-insensitive	Furosemide-sensitive component
NaCl	0.52 ± 0.02	0.26 ± 0.02	0.47 ± 0.03	0.22 ± 0.02
NaBr	0.39 ± 0.05[c]	0.15 ± 0.02[b]	0.45 ± 0.03[NS]	0.25 ± 0.01[NS]
Na acetate	0.10 ± 0.01[c]	−0.04 ± 0.03	0.29 ± 0.03[c]	0.06 ± 0.03[c]
$NaNO_3$	0.20 ± 0.01[c]	0.01 ± 0.02	0.42 ± 0.03[NS]	0.20 ± 0.04[NS]

[a] Each datum is the mean ± SE of three separate determinations. Significantly different from values in NaCl media: NS = not significant; [b] $P < 0.05$; [c] $P < 0.01$.

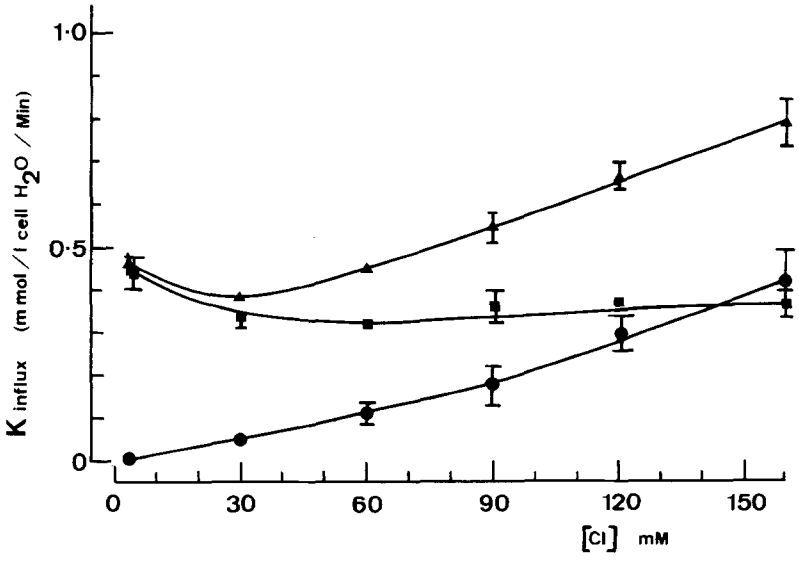

Fig. 10. Effect of medium Cl upon the ouabain-insensitive K^+ influx NaCl was replaced isosmotically by $NaNO_3$ in the presence (■) and absence (▲) of 0.1 mM furosemide. The furosemide-sensitive component (●) is the difference between the total ouabain-insensitive influx and the furosemide/oubain-insensitive influx. Preincubation conditions were identical to those of Fig. 8. Each point is the mean ± SE of three determinations

Action of Certain Pharmacological Agents

The action of furosemide and of piretanide are similar (Table 7). Ethacrynic acid (without cysteine) and phloretin are known to block the diuretic-sensitive pathway in other cells, but their affinities are reduced compared to furosemide. Similar results are also obtained for K^+ influx in MDCK cells where both agents partially block the furosemide-sensitive pathway (Table 7). The stilbene SITS is completely ineffective as an inhibitor of the diuretic-sensitive K^+ influx pathway. Amiloride at concentrations greater than that employed to blockade Na^+ transport in epithelia (Cuthbert & Shum, 1974) inhibits a significant proportion of the diuretic-sensitive K^+ influx (Table 7). 4,-aminopyridine, and 2,4,6-TAP also have a significant effect upon the diuretic-sensitive K^+ influx in MDCK cells.

Table 7. Fractional inhibition of the furosemide-sensitive K^+ influx by various pharmacological agents

Agent	Concentration (M)	Fraction inhibition (%)
Furosemide	1×10^{-4}	100
Piretanide	1×10^{-4}	100
SITS	1×10^{-5}	0
Ethacrynic acid	1×10^{-4}	53
Phloretin	1×10^{-4}	86
Amiloride	1×10^{-4}	59
2,4,6,-Triaminopyrimidine	1×10^{-3}	36
4,-aminopyridine	1×10^{-3}	56

[a] K influx was determined in ouabain-containing media (1×10^{-3} M) in the presence and absence of the drug and in the presence and absence of furosemide (1×10^{-4} M). All results are expressed in relation to the inhibition of K influx observed with 1×10^{-4} M furosemide. Each flux determination was performed in triplicate. Fractional inhibitions have 10 degrees of freedom.

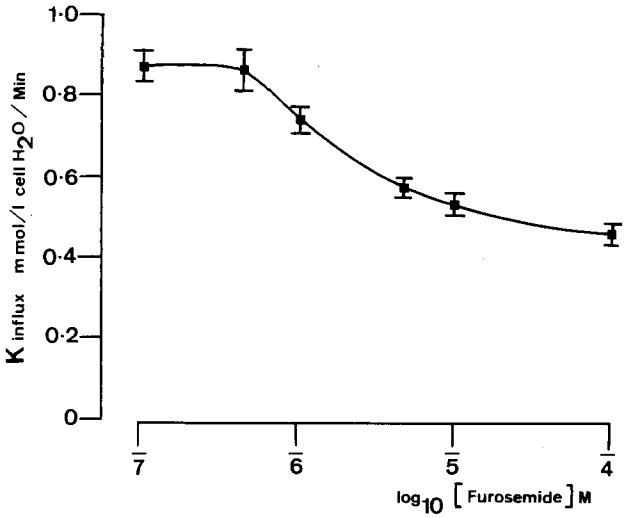

Fig. 11. Dose-response curve for the effect of furosemide upon the ouabain-insensitive K^+ influx (^{86}Rb as tracer). 50% reduction of the furosemide-sensitive component is seen at 3 μM furosemide. All determinations were performed in triplicate. Errors are ±SE of the mean

Effect of Ouabain, Furosemide and NO_3^- Replacement upon K^+ Efflux

Figure 12 demonstrates that the fractional K^+ loss in control Krebs' is time independent, consistent with K^+ loss from a single intracellular locus with no diffusion restriction in the extracellular space. This contrasts to basal loss of K^+ from epithelial layers grown on filters (Fig. 5) where filter thickness limits K^+ diffusion. 1×10^{-3} M ouabain causes a progressive increase in the fractional loss of K^+ (Fig. 12), which may result from recapture of ^{86}Rb in control conditions. Alternatively, this could represent a Ca^{2+}-stimulated K^+ leak pathway (Brown & Simmons, 1981). Externally applied furosemide, in contrast, results in a prompt inhibition of fractional K^+ loss; similarily, replacement of medium Cl^- by NO_3^- also results in a reduction in the fractional K^+ loss, but this is progressive (Fig. 12). The similar action of furosemide and of Cl^- replacement by NO_3^- parallels the observed effects upon K^+ influx (*see* above).

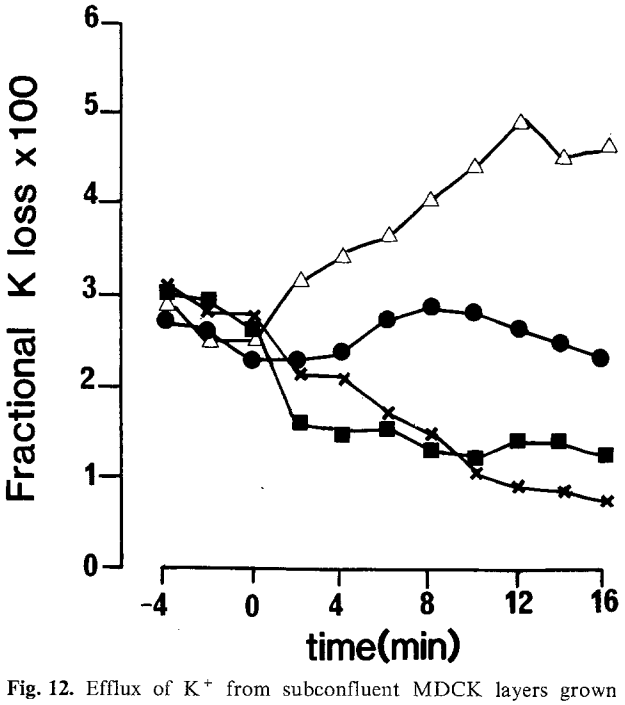

Fig. 12. Efflux of K^+ from subconfluent MDCK layers grown upon petri dishes. The first three washings were discarded. At time zero the wash solutions were changed from control solutions to experimental ones; (●) control, (△) 1×10^{-3} M ouabain, (■) 1×10^{-4} furosemide, (×) Cl^--free NO_3^- media. Data are taken from a single representative experiment

Discussion

The usual conceptual framework for active transepithelial cation transport is derived from the Kofoed-Johnsen und Ussing model (1958) for active Na^+ transport across frog skin; Na^+ diffusion into the cell across the apical membrane is coupled to active Na extrusion across the basal-lateral cell membranes by the $(Na^+ - K^+)$-ATPase. By analogy, transepithelial K^+ secretion would occur providing that the electrochemical gradient across the apical membrane was directed towards the apical solution. For MDCK epithelium the absence of net K^+ secretion, despite the existence of a functional $(Na^+ - K^+)$-ATPase located along the lateral interspace membranes (Simmons, 1981*a*; Lamb et al., 1981) implies a functional impermeability to K^+ for the apical cell membranes. Experimental data consistent with this are (i) the isotope ratio of ^{86}Rb: ^{42}K within the tissue after a 2-hr incubation and (ii) the measured K^+ influx and efflux across the apical cell membranes. The observed pattern of low apical K^+ permeability and relatively high basal-lateral permeability is consistent with measured ratios of apical to basal-lateral membrane resistances in other epithelia displaying high electrical resistance (Lewis, Eaton, Clausen & Diamond, 1977; Fromter & Gebler, 1977).

In addition to generating transepithelial Na^+ transport, the $(Na^+ - K^+)$ ATPase is also responsible for the regulation of intracellular cation levels. In various epithelia, cell potassium activities are invariant despite large changes in the overall rate of transepithelial Na^+ transport (Nellans & Schultz, 1976; Giebisch, 1979). Providing that the stoichiometry of the $(Na^+ - K^+)$-ATPase is invariant (Jorgenson, 1980), these findings imply the existence of transport mechanisms for K^+ other than the $(Na^+ - K^+)$ pump which match K^+ permeability to varied $(Na^+ - K^+)$-pump activity. For MDCK epithelium three components of K^+ influx across the basal-lateral membranes exist: firstly, a ouabain-sensitive component whose features closely resemble the known behavior of the $(Na^+ - K^+)$-pump mediated K^+ flux; secondly, an influx component sensitive to inhibition by furosemide, and, thirdly, a ouabain and furosemide-insensitive K^+ influx. There are also furosemide-sensitive, and insensitive components to K^+ efflux.

The furosemide-sensitive component of K^+ influx displays several characteristics similar to a facilitated mode of K^+ transfer, distinguishing this pathway from a simple passive leak pathway: (i) saturation kinetics, (ii) dependence upon Na^+ and K^+ and Cl^-, and (iii) inhibition by diuretics such as furosemide, piretanide and ethacrynate. The ouabain and furosemide-insensitive K^+ influx, in contrast, behaves as a simple passive leak pathway.

Although we have not specifically addressed the question, it is likely that the furosemide-sensitive component of K^+ influx is not involved in active uphill K^+ accumulation within the cell; thus, although K^+ influx is markedly reduced by furosemide, K^+ accumulation is not reduced by furosemide despite a 2-hr incubation; also ouabain inhibition in the absence of Na^+ results in a dissipation of pre-existing K^+ gradients.

The furosemide-sensitive component of basal-lateral membrane flux consists primarily of K:K exchange; thus external furosemide inhibits K efflux by 50%. However, there is some evidence that the furosemide-sensitive component may mediate some net K efflux from the cell across the basal-lateral membranes, as furosemide slightly increases K^+ concentrations with prolonged incubations.

A more precise knowledge of the function of the furosemide-sensitive K^+ pathway requires the behavior of the furosemide-sensitive K^+ efflux to be defined. In particular, knowledge of the dependence upon internal Na^+ and Cl^- concentrations would be extremely interesting. An activation of the K^+ efflux by Na over the range 10–30 mM could provide a mechanism whereby the basal-lateral membrane K^+ permeability may be linked to a parameter dependent upon net Na^+ transport. Clearly, studies of furosemide-sensitive K^+ transport are needed at elevated transepithelial Na^+ transport rates.

The furosemide-sensitive K^+ pathway, described here, shares many similar features to the $Na^+ - K^+$ Cl^- cotransport described for human red cells (Chipperfield, 1980, 1981; Dunham et al., 1980) ascites tumor cells (Geck et al., 1978, 1980), and avian erythrocytes (Bakker-Grunwald, 1981). Thus the K^+ influx is dependent upon medium Cl^-, with Br^- being partially able to support the K^+ influx. Also the furosemide-sensitive K^+ influx is dependent upon medium Na^+ (Dunham et al., 1980), Li^+ being partially able to substitute for Na^+.

The pharmacological sensitivity of the $Na^+ + K^+ + Cl^-$ cotransport system is identical to that observed here for K^+ influx; thus furosemide is one of several diuretics that may inhibit. Furosemide-sensitivity is of high affinity (3 μM). Phloretin also inhibits the furosemide-sensitive cotransport and K^+ influx (Chipperfield, 1981). The stilbene, SITS, is ineffective in MDCK cells upon K^+ influx similar to cotransport in red cells (Dunham et al., 1980).

The parallels between the $Na^+ + K^+ + Cl^-$ cotransport in other tissues and the K^+ influx observed here suggests that measurements of coupling between Na^+, K^+ and Cl^- fluxes across the basal-lateral membrane will be of interest. In this respect Rindler, McRoberts and Saier (1980) report preliminary results for both furosemide-sensitive Na^+ and K^+ in-

fluxes. Since the furosemide-sensitive cotransport may be responsible for Cl^- accumulation within a variety of cells (Chipperfield, 1980, 1981; Geck et al., 1980), the action of furosemide upon MDCK cytosolic Cl^- activities is also of interest. Linkage of the various passive ion fluxes across the basal-lateral membranes by a furosemide-sensitive transport pathway raises the possibility of a variety of transport modes being displayed by a single system, e.g., coupled NaCl influx, Na:Na exchange, KCl efflux, etc., as has been speculated for red cells (Chipperfield, 1980, 1981). We have recently identified a net transepithelial Cl^- secretion in MDCK epithelia which is inhibited by furosemide (Simmons, 1979); clear parallels between the diuretic-sensitive K^+ influx pathway demonstrated in this paper and the net Cl^- secretion would give credence to the participation of a $Na^+ + K^+ + Cl^-$ cotransport, similar to that in other tissues, in transepithelial Cl^- transport.

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