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Ion Transport in 'Tight' Epithelial Monolayers of MDCK Cells

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Summary. Cultured monolayers of MDCK cells grown upon filter supports display many features of *in vivo* epithelia. Previously reported values of transmonolayer resistance of $100\,\Omega cm^{-2}$ (Misfeldt, Hamamoto & Pitelka, 1976; Cereijido, Robbins, Dolan, Rotunno & Sabatini, 1978) indicate a leaky epithelium. This paper describes the properties of a strain of MDCK cells which displays entirely different electrophysiological properties. The results show that (i) the mean transmonolayer resistance is 4.16 $k\Omega\ cm^{-2}$, (ii) transmonolayer ion transport is of small magnitude since the mean spontaneous open circuit PD is only 2.17 mV basal surface positive and isotopic Na and Cl flux measurements fail to demonstrate a significant net flux, (iii) the action of ouabain, amiloride and ion substitutions are consistent with transmonolayer net Na movement being largely responsible for the spontaneous PD, and (iv) asymmetry in the localization of the Na–K ATPase is evident on the basis of ^{3}H-ouabain binding to cell monolayer.

Handler, Perkins and Johnson (1980) have directed attention towards the use of cell culture techniques in the study of epithelial transport function particularly with regard to renal tissue. That this is a serious possibility is derived from observations that epithelial cells from various organs form sheets of cells in culture which retain structural and functional characteristics similar to their tissue of origin (Misfeldt et al., 1976; Cereijido et al., 1978; Goldring, Dayer, Ausiello & Krane 1978; Handler, Steele, Sahib, Wade, Preson, Lawson & Johnson 1979; Handler et al. 1980).

An established cell-line, initially derived from dog kidney (MDCK), has been extensively studied in this respect. MDCK cells form monolayers of cells which possess a typical epithelial structure (Misfeldt et al., 1976; Cereijido et al., 1978; Cereijido, Ehrenfeld, Meza & Martinez-Palomo, 1980*a*) and which demonstrate net transmonolayer transport when grown upon permeable substrates (Misfeldt et al., 1976; Cereijido et al., 1978, 1980*a*). Retention of differentiated characteristics in this cell line is considerable since Rindler, Chuman, Shaffer and Saier (1979) have demonstrated an adenyl cyclase which is stimulated by renal-related hormones. Additionally, a cytoplasmic receptor protein for mineralocorticoids has been characterized (Ludens, Vaughn, Mawe & Fanestil, 1978).

Surprisingly, little information is available regarding the nature and mechanism of transmonolayer ion transport in MDCK cells. Thus, passive permeability properties of the MDCK epithelium are well-characterized (Misfeldt et al., 1976; Cereijido et al., 1978, 1980*a*) and indicate that MDCK epithelium is of the low resistance type (80–100 Ωcm^{-2}). Net ion flux measurements remain unconvincing (Cereijido et al., 1980*a*) primarily due to the difficulties in measuring small net ion fluxes in the presence of large bidirectional fluxes. An additional complication is the recent finding, made in this laboratory (Barker & Simmons, 1979) that different strains of MDCK cell exist which display entirely different physiological properties. In particular we have found that MDCK cells of between 60 and 66 serial passages from high-resistance epithelial monolayers of $\sim 4\,k\Omega\,cm^{-2}$. Resistance values similar to previously reported values are recorded, but only in monolayers formed from cells of between 110 and 116 serial passages.

The purpose of this paper is twofold: Firstly, to emphasize the existence of MDCK cells which form "tight" epithelial monolayers, and secondly to explore the magnitude and nature of transepithelial ion transport in these monolayers.

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Materials and Methods

Cell Culture

MDCK dog kidney cells were obtained from Flow Laboratories (Irvine, Scotland) at 60 serial passages. The cells were maintained in serial culture in Roux Flasks in Minimum Essential Medium Eagles (MEME) supplemented with 10% vol/vol fetal bovine serum, 1 unit/ml gentamycin antibiotic, nonessential amino acids and 2 mM glutamine (Flow Laboratories) at 37 °C in an air 5% CO_2 atmosphere. Cells were subcultured by trypsinization in a Mg, Ca-free Earles salt solution with 2 mM EDTA (disodium salt). Source stocks of cells, from which all subsequent passages are made, are held in a liquid nitrogen store in complete MEME growth medium with 10% vol/vol dimethyl sulfoxide as cryoprotectant. Cell monolayers were prepared on 2.5 cm diameter Millipore membrane filters (0.22 μm pore diameter) by seeding at high density (Cereijido et al., 1978) followed by growth to confluency in complete MEME with 1 IU/ml insulin (Boots Chemical Co.) as an additional growth stimulant (Misfeldt et al., 1976). Cell monolayers were used for resistance/PD measurements after 3 to 4 days. Checks on monolayer confluency were made by microscopic examination of fixed dehydrated monolayers whose millipore support had been made transparent with toluene.

Solutions and 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. The use of saturated KCl minimized error due to liquid junction potentials. In view of the small potential differences measured under open-circuit conditions an extra precaution was introduced to aid accuracy; the zero of the calomel cells and their salt bridges was checked before each PD measurement by placing the KCl-agar leads together in the same half-chamber of the Ussing apparatus. All PD's are expressed with relation to the basal surface of the cell layer attached to the millipore filter (the serosal surface). The apical surface is therefore the side of the cell layer facing away from the millipore filter. Resistance determinations were made routinely by passing 2 μA hyper-polarizing current pulses across the cell monolayer except where current was varied. Constant current conditions were ensured by including a series 300 kΩ resistor. Current pulse duration was controlled by a feedback signal generator. Current was measured by the potential drop across a precision 1 kΩ resistor placed in series. For fast sweeps (0 to 200 msec) of voltage deflections a Data Lab DL90 single-channel transient recorder (Surrey U.K.) was used for recording. This device allows digitization of the record with 1,000 data points with slow playback to a chart recorder. The time resolution of this fast recording is 0.2 msec. The rise-time of square current and voltage pulses passed across the Ussing chamber without an epithelial monolayer recorded using an oscilloscope was 30 μsec. The experiments were invariably carried out at pH 7.4 in modified Krebs solution containing (mmol/liter): NaCl, 137; KCl, 5.4; $CaCl_2$, 2.8; $MgSO_4$, 1.2; NaH_2PO_4, 0.3; KH_2PO_4, 0.4; HCl, 12; Tris base, 14; glucose, 10; glutamine, 2; Na pyruvate, 2; 2% vol/vol fetal bovine serum and amino acids for Eagles medium (Flow Laboratories).

Na Ion Flux Measurements

Bidirectional Na ion fluxes were determined simultaneously on the same cell monolayer under voltage clamp using ^{22}Na and ^{24}Na as tracers (Simmons & Naftalin, 1976) obtained from the Radiochemical Centre, Amersham, U.K. Solutions were not gassed due to the inclusion of 2% vol/vol fetal bovine serum. Two consecutive flux periods were for 30 min, total chamber volumes 14 cm^3 and sample volumes 1 cm^3; ^{24}Na was counted by its Cherenkov emissions in a Packard liquid scintillation counter. ^{22}Na was counted after ^{24}Na decay (10 half-lives) by its β-emissions in a Packard liquid scintillation spectrometer.

Cl Ion Flux Measurements

Conditions were identical to those for Na ion measurements. Cl fluxes were measured using ^{36}Cl as tracer. The two bidirectional fluxes were determined upon the same monolayer using a randomized order to avoid systematic error. ^{36}Cl activity was measured by its β-emissions in a Packard liquid scintillation spectrometer.

[^{3}H]-Ouabain Binding

[^{3}H]-ouabain binding was determined in MDCK cells grown upon millipore filters. Specific binding of [^{3}H]-ouabain was determined using incubations in a K^+-free medium and one containing 15 mM K^+ (Baker & Willis, 1972). Since K^+ alters the rate of ouabain binding and not the total binding, both the time and concentration dependence of [^{3}H]-ouabain binding were determined in K-free and 15 mM K^+-containing media (Barker, Lamb, Ogden & Simmons, 1978). The apparent K_m for specific [^{3}H]-ouabain binding was 8.0×10^{-8} M and equilibrium values of 'specific' binding were observed by 20-min incubation (Barker et al., 1978). [^{3}H]-ouabain binding was determined using a 20-min incubation with a ouabain concentration of 2×10^{-7} M in K^+-free and 15 mM K^+ media.

Asymmetric labeling was performed by clamping cells in Ussing chambers maintained at 37 °C and adding ^{3}H-label to either bathing solution. The exposed area of cell monolayer was 1.76 cm^2. The bathing solution was the modified Krebs (*see above*). Monolayers were washed four times with ice-cold Krebs prior to extraction in triton/toluene. Cell numbers were measured on separate monolayers by releasing cells by trypsin-EDTA and then counting using a Coulter-Counter. MDCK monolayer integrity was checked by resistance measurements using 2 μA/cm^2 hyperpolarizing current pulses.

Electron Microscopy

Cell monolayers were fixed in 2% glutaraldehyde in MEME (pH 7.4) for 1 h, washed in 112 mM Na cacodylate buffer and then post-fixed in 1% osmium tetroxide in 112 mM cacodylate buffer for 1 h. The millipore filters and attached monolayers were then dehydrated in an ascending series of alcohols before being finally cleared in toluene; they were subsequently embedded in araldite and thin sections were cut using glass knives on an OmU_2 microtome. Thin sections were viewed on a Philips electron microscope after staining with lead citrate and uranyl acetate.

Chemicals

All chemicals were of ANALAR grade. Amiloride was a gift from Merck Sharp and Dohme. The stilbene 4, acetamido, 4′-isothiocyano-2′, 2′ stilbene disulfonic acid (SITS) was obtained from BDH Chemicals and added as an ethanolic solution. Furosemide was from Hoechst.

Statistical Methods

Variation in results is expressed as the standard error of the mean. Tests for significance of differences were made by a two-tailed Student's *t*-test (unpaired means solution). One-tailed tests and paired tests were used where appropriate. Skewness of monolayer PD and resistance distributions was assessed by the coefficient of skewness whose significance is tested against 0 by a one-tailed *t*-test (Snedecor & Cochran, 1968).

Results

Mean Values of Resistance and Open-Circuit PD

Shown in Figs. 1 and 2 are histograms illustrating the magnitude of resistance and spontaneous open circuit PD recorded from monolayers of MDCK cells of between 60 and 66 serial passages. In contrast with previously reported values for the resistance of MDCK epithelial monolayers (80–100 Ω cm^{-2}, Misfeldt et al., 1976; Cereijido et al., 1978, 1980*a*) the mean resistance is 4.1 kΩ cm^{-2}. The distribution of resistances is markedly skew towards higher resistance values ($P<0.01$ for a significant coefficient of skew). The mean open-circuit PD is 2.17 mV, basal surface positive and again the distribution of PD's is skew towards higher values of PD. The mean short-circuit current across these monolayers is therefore small $\sim$0.53 μA cm^{-2}. Due to the high mean transmonolayer resistances recorded in 61–66 passage monolayers it is likely that edge damage due to compression of the monolayer in the Ussing chamber will affect these measurements (Helman & Miller, 1973). The effect of such damage is to introduce a shunt pathway for ion movement in parallel to the epithelium. The presence of such damage is evident from the distribution of both resistance and PD in 61–66 passage monolayers, which are markedly skew towards higher values and from the direct linear correlation between resistance and PD (correlation coefficient $r=0.615$, $n=232$, $p<0.01$; Fig. 3). This latter correlation is of particular significance since urinary bladder, mounted so as to reduce edge damage to a minimum, shows an inverse correlation between PD and resistance (Higgins, Gebler & Frömter, 1975). The mean values of transmonolayer resistance and PD in this set of data are thus probably underestimates of their true values. In view of this fact I have attempted to select data from epithelial monolayers which display higher values of resistance and open-circuit PD in the belief that such monolayers will reflect the true behavior of this epithelium.

Current-Voltage Relationship

Tight epithelia are known to show time-dependent effects in conductance with transepithelial current

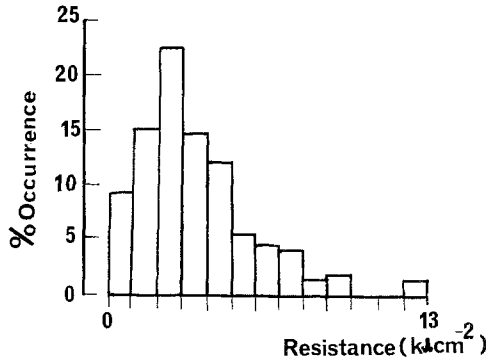

Fig. 1. Histogram of the transmonolayer resistance recorded 5 to 10 min after mounting cell monolayers in the Ussing chamber. The mean resistance of the epithelial monolayers was 4,116.9 $\pm$ 238.6 Ω cm^{-2} (SEM), $n=232$). Coefficient of skewness $=3.89\pm0.16$ ($p<0.01$). The class width of this histogram is 1 kΩ cm^{-2}

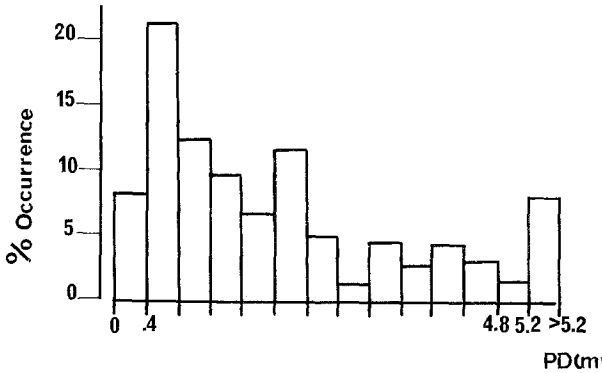

Fig. 2. Histogram of the spontaneous open circuit PD recorded 5 to 10 min after mounting cell monolayers in the Ussing chamber. The mean PD was 2.17 ± 0.13 mV (SEM), coefficient of skeweness $=$ 1.68 ± 0.16 ($p<0.01$). The class width is 0.4 mV

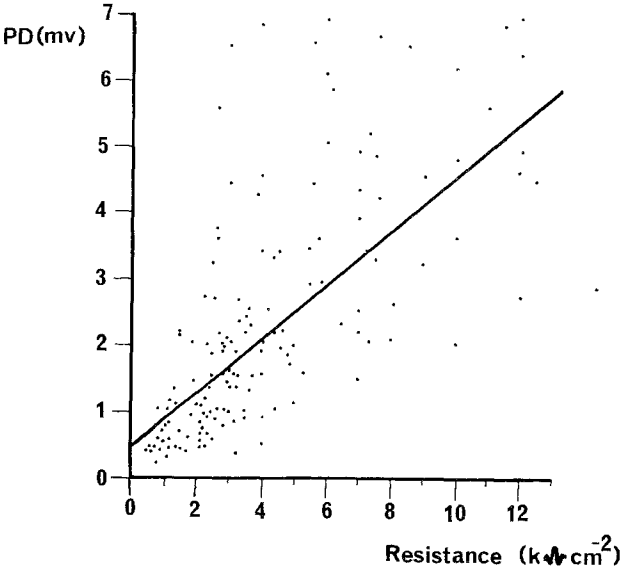

Fig. 3. Scatter diagram showing the relationship between resistance and transmonolayer PD in the data from Figs. 1 and 2. The solid line is the least-squares line. $y=0.48\pm0.17$ (SE) $+4.13\pm0.35\times10^{-4}x$ (SE). The slope is significantly different from zero ($P<0.01$) and the correlation coefficient is 0.62 ($P<0.001$)

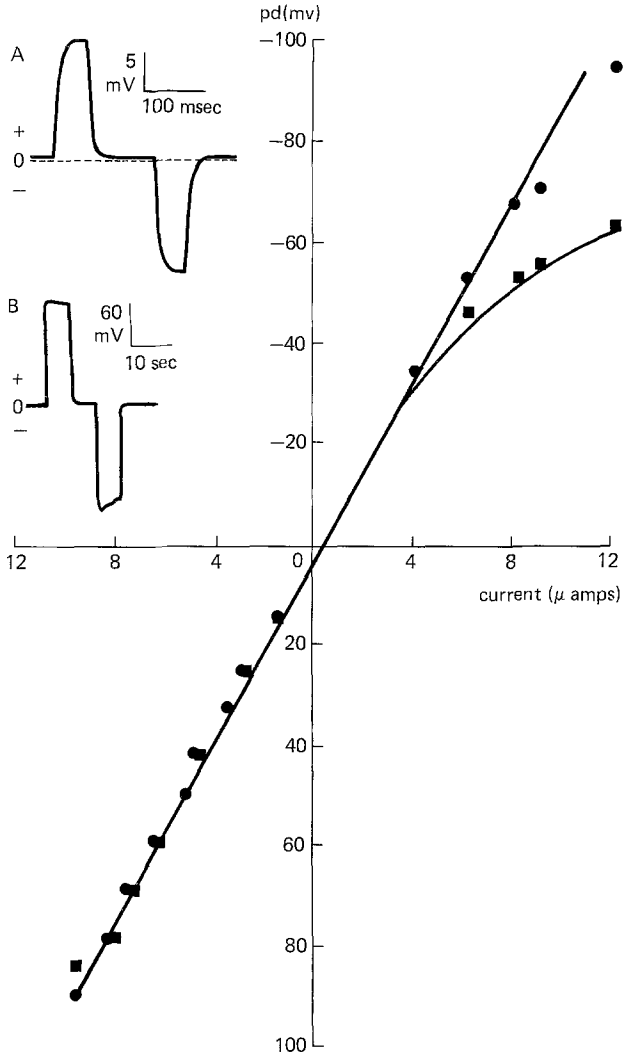

Fig. 4. Current-voltage relationship of high-resistance MDCK epithelial monolayers. Insets *A* and *B* show voltage transients recorded at two different current densities (4.5 and 45 μA cm^{-2}, respectively) and sweep speeds from a single experiment. Constant current pulses had a rise-time of 30 μsec. The current-voltage diagram was obtained in a single experiment. Abscissa: transepithelial current in μA cm^{-2}; ordinate: transepithelial potential difference in mV (basal bathing solution, − or +). ●, values recorded 0.5 sec after current onset; ■, values recorded 50 sec after onset of current flow. Nonzero intercept on PD axis is the small spontaneous PD

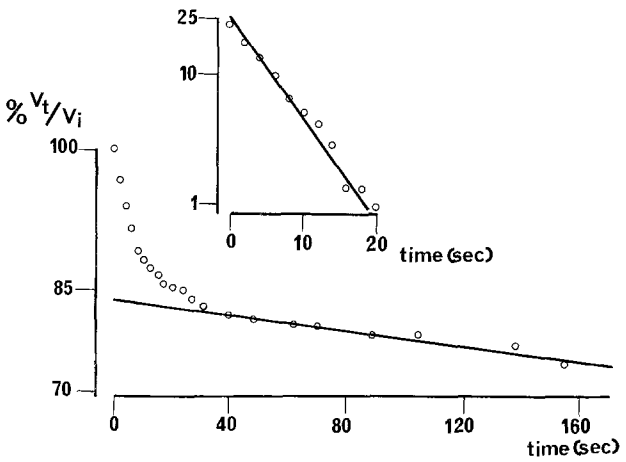

Fig. 5. Semi-log_e plot of the decrease in voltage expressed as the percentage of the initial voltage deflection during a prolonged depolarizing current pulse of 9 μA cm^{-2} for 0–170 sec. The decay in voltage can be approximated by the sum of two exponentials of time constants 6.12 and 108 sec^{-1}. Solid lines give the least-squares lines. Both correlation coefficients were <0.98 ($n=10$ and 8, respectively)

flow (Bindslev, Tormey, Pietras & Wright, 1974). However, with low current densities and after the capacitive surge the current-voltage relationship for toad urinary bladder is linear up to ±350 mV (Bindslev et al., 1974).

The insets to Fig. 4 (*A* and *B*) show voltage transients across an epithelial monolayer of MDCK cells following application of a square-wave constant current pulse (rise-time = 30 μsec) at low and high current densities with two sweep speeds. At a current density of 4.5 μA cm^{-2} (*A*) the initial rise in voltage is approximated by a single exponential which corresponds to a tissue capacitance of 0.9 μF/cm^{-2}. The plateau value of voltage after 50 msec gives a resistance of 3.1 kΩ cm^{-2}. At low current densities the plateau value of voltage is maintained with longer current pulse duration (up to 50 sec). With higher current densities (Fig. 4*B*) initial values of voltage at 0.5 sec also give a resistance of 3.1 kΩ cm^{-2}. The slope resistance recorded at 0.5 sec is linear in the range ±100 mV. [Figure 4 current-voltage diagram was recorded in a different experiment where the monolayer resistance is high (9.64 kΩ cm^{-2}).] With depolarizing current pulses there is a marked time-dependent increase in conductance (Fig. 4, inset B and current-voltage diagram) in the range of −30 to −100 mV. An interesting feature of this increased conductance is the asymmetric nature of this effect; hyperpolarizing current application, within the range used, causes relatively small increments in conductance. Two features of this increased conductance are similar to those described by Bindslev et al. (1974). (i) The increased conductance is rapidly reversed since repetitive current pulses (50-sec interval) give equivalent voltage deflections; (ii) the larger the current density the greater the increased conductance. (Transepithelial voltage values seem to be more important as a determinant, however, since relatively smaller conductance changes are seen in Fig. 4, inset *B* where monolayer resistance is only 3.1 kΩ cm^{-2}). The increased conductance seen with depolarizing current pulses is retained when all Na in the bathing Krebs is replaced by choline and when Cl is replaced by isethionate and SO_4 (with mannitol to maintain osmolarity) (*data not shown*). The increase in ionic conductance is therefore generalized and may not represent a selective pathway for ion flow. A candidate

Table 1. Ion fluxes across MDCK epithelia

Ion and concentration (mM/l)	n	J_{A-B} μmoles cm^{-2} hr^{-1}	P_{A-B} cm^{-1} hr^{-1} $\times 10^3$	J_{B-A} μmoles cm^{-2} hr^{-1}	P_{B-A} cm^{-1} hr^{-1} $\times 10^3$	J_{net} μmoles cm^{-2} hr^{-1}	Short-circuit current μmoles cm^{-2} hr^{-1}	Monolayer conductance mMho cm^{-2}
Na (137)	17	0.89±0.09	6.5±0.7	0.71±0.03	5.2±0.25	0.20±0.16	0.036±0.001	1.011±0.074
Cl (160)	19	0.81±0.16	5.1±1.0	1.00±0.25	6.25(5)±1.5	−0.31±0.27	(n=36)	(n=36)

All ion fluxes were determined over 2½ hr periods under voltage clamp. J_{A-B} is the flux of an ion from apical to basal bathing solutions. Permeabilities were calculated from the relationship $P_{A-B}=J_{A-B}/C_A$ where C_A is the concentration of the ion in compartment A

pathway may be the junctional route as discussed by Bindslev et al. (1974). The increase in conductance with time is not a simple single exponential process (Fig. 5). The decay in voltage with time during a prolonged depolarizing current application is approximated by the sum of two exponentials of time constants 6.12 and 108 sec^{-1}. Small solute polarization effects may be of importance to an interpretation of the bi-exponential nature seen with prolonged current applications (Wedner & Diamond, 1969); and also with the decay of voltage observed following high current densities (Fig. 4, inset *B*). The nature of the current-voltage relationship contrasts with that observed in low-resistance monolayers (Misfeldt et al., 1976; Cereijido et al., 1978).

Magnitude and Nature of Ion Transports in 60–66 Passage Monolayers

I have attempted to enhance the accuracy of flux determinations in the following manner: (i) bidirectional Na-ion fluxes were determined simultaneously upon the same epithelial monolayer using ^{22}Na and ^{24}Na as tracers for Na; Cl fluxes were determined using ^{36}Cl only, but measurements were on the same monolayer, (ii) the exposed monolayer area was 1.76 cm^2; (iii) flux measurements were performed over half-hour periods, though isotope equilibration was never greater than < 1%. The current-flux equivalent recorded over the flux measurement periods was 0.036 μmoles cm^{-2} hr^{-1} (Table 1). This value is within the errors observed in flux measurements (Table 1) and would be impossible to detect if this represented total net ion movement.

The net fluxes of both Na and Cl are in fact not significantly different from zero ($P>0.1$ in both cases) (Table 1). It is, therefore, impossible to assign the spontaneous open-circuit PD to the movement of a particular ion on the basis of isotopic ion flux measurements. Table 1 shows that the magnitude of the unidirectional ion fluxes of Na^+ and Cl^- are similar, but are small compared to previous measurements (Cereijido et al., 1978, 1980*a*). The unidirectional flux data is consistent with the high electrical

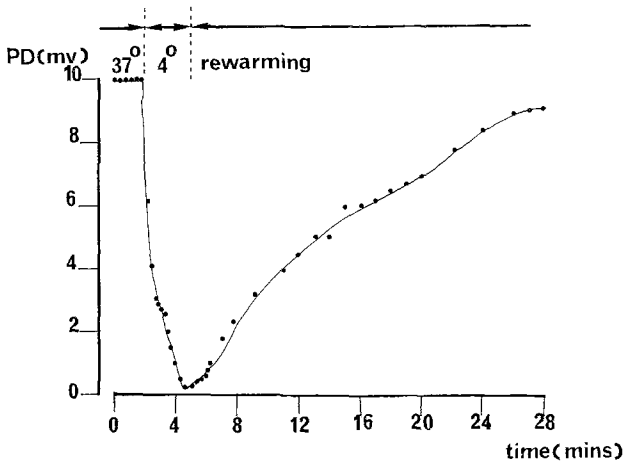

Fig. 6. Effect of a temperature reduction upon the spontaneous PD maintained by an MDCK monolayer of resistance 8.2kΩ cm^{-2}. To reduce the temperature of the thermostated block, circulation fluid was switched from a bath at 37 °C to one at 4 °C, simultaneously the Krebs solution bathing both sides of the cell monolayer was replaced with a cooled Krebs at 4 °C. Rewarming was attained by switching back to 37 °C circulation fluid but with no replacement of Krebs

resistance of 60–66 passage monolayers reported here (Table 1, Fig. 1).

In order to explore the ionic basis of the spontaneous PD I have resorted to various experimental manipulations including application of drugs known to inhibit transepithelial transport in other tissues (Figs. 6, 7, 8 and Table 2).

Cooling of the cell monolayer to 4 °C results in an abolition of the basal surface positive PD indicating its ultimate dependence upon active transport (Fig. 6). Addition of ouabain (1×10^{-4} M) to the apical bathing solution has no effect upon the spontaneous PD (Fig. 7) whereas addition of an equivalent concentration to the basal bathing solution results in a steady decline in the basal surface positive PD to zero. Since the known action of ouabain is to inhibit the Na−K ATPase in this tissue (Abaza, Leighton & Schultz, 1974) this result implies that the PD is entirely dependent upon transmonolayer Na^+ transport and that the Na−K ATPase is localized exclusively on the basal surfaces of this cell layer

in accord with direct autoradiographic localization (Barker et al., 1978). It should be noted, however, that basal application of ouabain causes a drop in monolayer resistance from 7.8 $k\Omega\,cm^{-2}$ (prior to ouabain application) to 4.2 $k\Omega\ cm^{-2}$ at 45 min (Fig. 7) indicating nonspecific actions of Na–K pump inhibition.

Amiloride is known to reduce transepithelial Na transport in a variety of tissues (Cuthbert & Shum, 1974); apical amiloride promptly reduces the open-circuit PD (basal surface positive) from 6.8 to 5.5 mV (basal surface positive) and increases transepithelial resistance (Fig. 8). 54% of the short-circuit current (SCC=PD/resistance) is, however, amiloride insensitive. This result is confirmed in Table 2, where on ten separate preparations, an amiloride inhibition of the basal surface positive PD is observed. A large amiloride-insensitive component of PD exists and effects on transmonolayer resistance are not consistently observed. The absence of large effects of amiloride on transmonolayer resistance (Table 2) is consistent with the low PD and low net transepithelial Na flux recorded here (*see also* Discussion). These results are similar to those found by Cereijido et al. (1978, 1980*a*) for low-resistance monolayers of 100–110 passages.

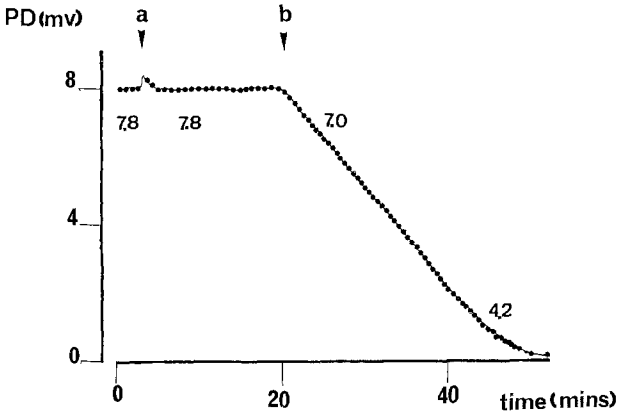

Fig. 7. Effect of ouabain upon the spontaneous PD. At *a*, 1×10^{-4} M ouabain was added to the apical (cellular) bathing solution; at *b* the same concentration of ouabain was added to the basal bathing solution. The Krebs was of normal composition containing 5.4 mmol/liter KCl. Figures indicate determinations of the transmonolayer resistance at the various periods of the experiment

Inhibitors of anion transport such as the stilbene derivative SITS (Cabantchik, Knauf & Rothstein, 1978) or furosemide (Degnan, Karnaky Zadunaisky, 1977; Silva, Stoff, Field & Fine, 1977) are without effect on the basal surface positive spontaneous PD either alone or in combination with amiloride (Fig. 8). A similar negative result has been obtained with phloretin (*unpublished results*).

Replacement of bathing solution Na by choline reduces the spontaneous PD and monolayer conductance (Fig. 8 and Table 2). In twenty separate determinations 91% of the SCC is Na-dependent.

Replacement of bathing solution Cl for the impermeant anions isethionate and sulfate reduces the open-circuit PD (Fig. 8 and Table 2). Cl-free solutions result in a pronounced decline in monolayer resistance which is not reversed upon return to control bathing solutions (Table 2). This nonspecific effect of Cl^- substitution upon monolayer conductance is likely to explain the difference in resistance observed for

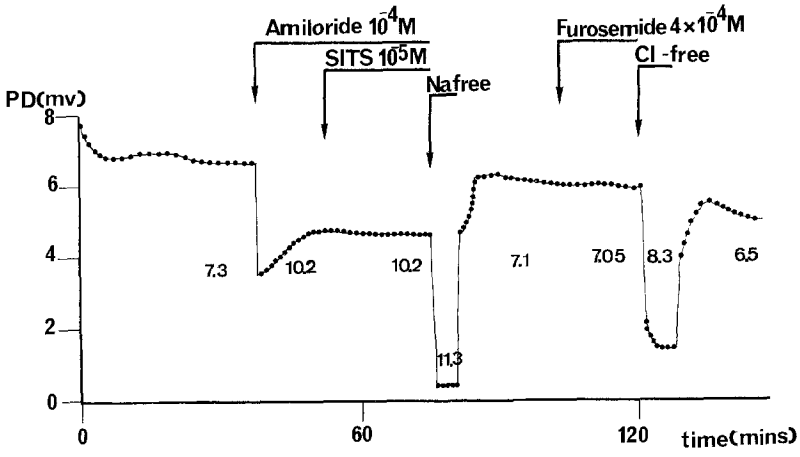

Fig. 8. Effect of inhibitors of ion transport and of ionic substitutions upon the spontaneous PD. Amiloride was added to the apical solution only, the stilbene derivative, 4-acetamido,4′isothiocyano-2,2′stilbene disulfonic acid (SITS) and furosemide were added to both bathing solutions. The Na-free solution was attained by replacement of NaCl by choline Cl and omitting NaH_2PO_4. The Cl-free solution was obtained by replacing NaCl with Na isethionate while other Cl salts were replaced by the sulfate salts, mannitol being added to maintain isotonicity. Both bathing solutions were replaced. Serum used in ion replacement solutions was dialyzed overnight against two changes of ×20 volume distilled water. Dialyzed serum in no way affects the spontaneous PD as compared with undialyzed serum. Figures indicate determinations of the transmonolayer resistance at the various periods of the experiment

Table 2. Averaged data of separate determinations of the effect of ion substitutions and amiloride upon monolayer resistance and spontaneous potential difference

Treatment	n	Control$_1$[a]	Experiment	Control$_2$
Cl substitution by isethionate/SO_4	25	1.55 ±0.31 (mV) 3993±828 (Ω cm^{-2})	0.27 ±0.08** (mV) 3453±744 (Ω cm^{-2})	0.94 ±0.24 (mV) 1989±366 (Ω cm^{-2})
Na substitution by choline	20	2.59 ±0.65 (mV) 3991±683 (Ω cm^{-2})	0.31 ±0.12** (mV) 5007±1068* (Ω cm^{-2})	1.83 ±0.36 (mV) 3175±499 (Ω cm^{-2})
Mucosal amiloride (1×10^{-4} M)	10	2.85 ±0.58 (mV) 6475±1088 (Ω cm^{-2})	2.62 ±0.64* (mV) 6975±998 (Ω cm^{-2})	2.79 ±0.52 (mV) 5614±931 (Ω cm^{-2})

Control periods refer to determinations prior to (1) and subsequent to (2) treatment
[a] Significantly different from control$_1$ values (paired t-test):
* $P<0.05$.
** $P<0.01$.

resistance values recorded in the Na-free and Cl-free media (Table 2), since flux measurements (Table 1) imply that the partial ionic conductances for Na and Cl are similar. It is of interest to note that the Cl-insensitive SCC in Fig. 8 is similar to the amiloride-sensitive component in the same Figure.

Taken together the simplest interpretation of this data is that net transmonolayer ion transport is dominated by an apical-to-basal net Na movement, Na influx across the apical membrane consisting of an amiloride-sensitive component and a coupled Na – Cl influx.

^{3}*H-Ouabain Binding in MDCK Monolayers*

In order to assess the maximal capability of high-resistance MDCK cell monolayers to transport Na, the binding of ^{3}H-ouabain to cell monolayers has been measured (Table 3). Specific binding (assessed by the difference in binding between K^+-free and K^+-containing Krebs) was 3.21×10^5 sites/cell and was entirely confined to sites accessible from the basal surface in accord with the action of ouabain upon PD (Fig. 7). The number of cells per cm^2 of monolayer was 5×10^5 cells. The amount of specific ouabain binding to MDCK cells is of a similar order of magnitude to that observed in HeLa cells (Boardman, Lamb & McCall, 1972; Boardman, Huett, Lamb, Newton & Polson, 1974) and to that observed by Rabito and Tchao (1980) for low-resistance MDCK cells, but is one order of magnitude smaller than that observed by Cereijido et al. (1980*a*) for 100–110 passage cells.

Structural Appearance of High-Resistance MDCK Monolayers

Due to the extensive differences in characteristics reported here, particularly with regard to transepithelial resistance (Misfeldt et al., 1976; Cereijido et al., 1978, 1980*a*) thin sections (four preparations, ten grids per preparation) of araldite-embedded monolayers were examined to assess if differences were evident in cellular morphology. Figure 9 shows a typical portion of a monolayer of high-resistance MDCK cells. The overall structure and distribution of cytoplasmic constituents is similar to that described by Cereijido et al. (1978). 60 passage cells appear more flattened; however, cell height was only 3 to 3.5 μm compared with 10 μm observed by Misfeldt et al. (1976) or 7 μm observed by Cereijido et al. (1980*a*).

Extensive foldings and interdigitations are absent in the lateral spaces of high-resistance MDCK epithelia (Fig. 9). A similar absence of extensive lateral-space membrane amplification is also evident in low-resistance MDCK monolayers (Misfeldt et al., 1976; Cereijido et al., 1978, 1980*a*). Microvillus appearance in high-resistance monolayers differs substantially from the appearance of microvilli in low-resistance monolayers. In all sections viewed microvilli were short (0.2 μm in length) and stained heavily with lead citrate and uranyl acetate. A prominent glycocalyx is evident at high magnification (Fig. 9, inset). A stereological analysis of cellular dimensions in MDCK monolayers is at present being undertaken to quantify certain of the structural features described here.

Table 3. The number of Na–K pump sites in 60–66 passage MDCK cells

		No. of sites/cell $\times10^5$	Specific ^{3}H-binding sites/cell $\times10^5$
Basel labeling 0 K^+	6	5.37 ±1.06	3.21±0.929
Basal labeling 15 mM K^+	4	2.16 ±0.27	
Apical labeling 0 K^+	3	0.42 ±0.08	–
Apical labeling 15 mM K^+	3	0.479±0.05	

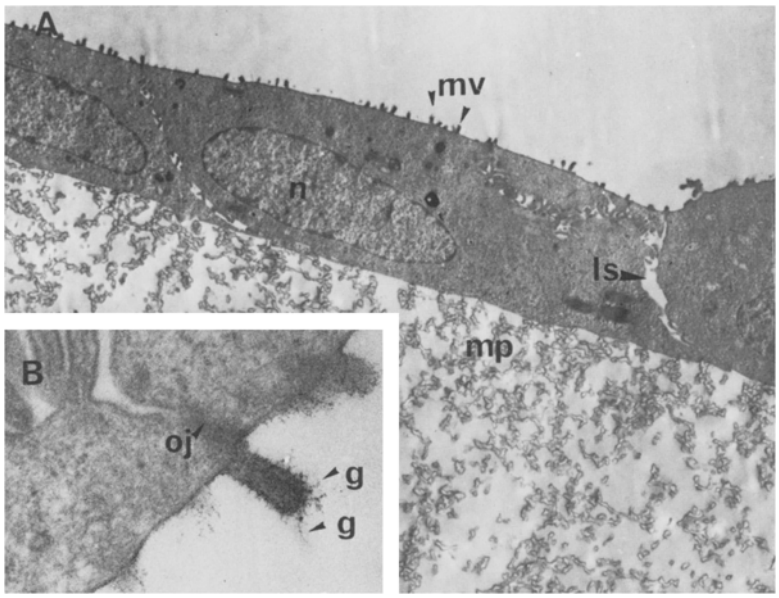

Fig. 9. Electron micrograph showing a portion of a typical monolayer of 60–66 passage MDCK cells growing on a millipore filter (5,500×). *mv* = microvilli; *ls* = lateral space; *n* = nucleus; *mp* = millipore substrate. Inset shows high power micrograph (58,400×) of junctional area. *oj* = occluding junction; *g* = glycocalyx

Discussion

Previous measurements of the electrophysiological parameters of MDCK epithelium (Misfeldt et al., 1976; Cereijido et al., 1978, 1980*a*, *b*) have demonstrated that this epithelium may be classified as a 'leaky' or low-resistance type. We have found this to be the case but only with monolayers formed from cells of 100 serial passages and above (Barker & Simmons, 1979). The present paper describes ion transport across monolayers of MDCK cells formed from cell stocks of between 60 and 66 serial passages. The mean transmonolayer resistance is 4.15 kΩ cm^{-2}, and although this is probably an underestimate of the true value due to edge damage, this result clearly places this epithelium in the high resistance or 'tight' category. This finding is substantiated by the nature of the current-voltage relationship (Fig. 4) and by the low bidirectional ion permeabilities observed for both Na and Cl in these epithelial monolayers (Table 1).

There exist, therefore, at least two different strains of MDCK cells which form epithelial monolayers of entirely different properties. This finding has implications for previous studies of MDCK epithelia in which it is assumed that the cell population is homogenous. Although subculture dependent changes in the properties of cultured cell-lines are known to occur (*see* Gilbert & Migeon, 1977) and low-resistance monolayers may simply be a dedifferentiated form of that present initially, it is also a possibility that the cell population is not entirely homogenous and that at least two cell types exist (overall monolayer electrophysiological properties thus depending upon the relative population frequency and junctional parameters of the two cell types). Cereijido et al. (1980*b*) have reported heterogeneity in junctional resistances between adjacent cells in low-resistance (100–110 passage monolayers. The voltage dependent/time dependent increases in conductance observed during current passage in urinary bladder epithelium have been interpreted in terms of change in junctional parameters (Bindslev et al., 1974), and it is of interest to point to the relative sensitivity of the cultured MDCK epithelium to applied currents. The possibility that junctional properties are dynamic and so easily perturbed is thus not excluded by present results.

Net ion transport across the high-resistance monolayers is indicated by the generation of spontaneous basal surface positive PD. However, net ion transport is small and cannot be measured accurately using isotopic flux techniques (Table 1). The range of net ion transports observed in cultured epithelia is shown in Table 4. The spontaneous PD of MDCK monolayers reported here, is sensitive to cooling, basal application of ouabain, apical amiloride and replacement of bathing solution Na and Cl by impermeant substitutes. This data is consistent with the spontaneous PD being largely generated by transepithelial Na movement, though alternative explanations are possible. To assess whether the low magnitude of net ion transport is the result of an intrinsic inability of these monolayers to generate net ion movement we have measured the number and distribution of [^{3}H]-ouabain binding sites by apical or basal application of [^{3}H]-ouabain (Cereijido et al., 1980*a*). The Na−K ATPase is accessible to ^{3}H-label only from the basal solution, supporting its localization by the abolition of the transmonolayer PD with basal application of

Table 4. Summary of the transepithelial resistances, short-circuit currents and net ion fluxes observed in cultured epithelia grown on permeable supports

Cell-line of primary culture	Resistance (Ω cm^{-2})	Short-circuit current (μmoles cm^{-2} hr^{-1})	Isotopic net flux (ion and magnitude) (μmoles cm^{-2} hr^{-1})
MDCK Low-resistance (100–110 passages)	80–100[a–d]	0.62[a] 0.28[b] 0.11[c]	– (Na) 2.7[d] –
MDCK High-resistance (this work)	4166	0.04	No significant net Na flux
LLC PK1	400 Ω[e]	–	–
TB-M	5000 Ω[f]	0.32[f]	(Na) 0.32[f]
TB-6C	10,000[f]	0.09(5)[f]	(Na) 0.10[f]
Primary mouse mammary epithelial cells	500[g]	0.95[g]	(Na) 0.9[g]

References

[a] Misfeldt et al. (1976).
[b] Cereijido et al. (1978).
[c] Barker and Simmons (1979).
[d] Cereijido et al. (1980*a*).
[e] Handler et al. (1980).
[f] Handler et al. (1979).
[g] Bisbee, Machen and Bern (1979).

ouabain (Fig. 7). The number of specific ouabain binding sites is 3.2×10^5 sites/cell (Table 3). Assuming that (i) 1 molecule of ouabain binds to one pump site, (ii) the maximal turnover number of the Na–K ATPase is 100 molecules of ATP split/site/second (Baker & Willis, 1972 and *unpublished observations*), (iii) 3 Na ions are pumped per ATP split, (iv) transepithelial Na transport is dependent upon the Na–K ATPase, and knowing the cellular density of the monolayer is 5×10^5 cell cm^{-2}, an upper limit for net Na transport is 0.28 μmoles cm^{-2} hr^{-1} or approximately 8 μA cm^{-2}. Although this estimate is subject to considerable error, it seems reasonable to suggest that transepithelial ion transport of Na in these monolayers is effectively limited by Na ion diffusion across the apical membrane. The low potential/high resistance profile characteristic of the MDCK layers observed here is evident in other cell-lines, in particular those isolated from toad urinary bladder (*see* Table 4) and is similar to natural epithelia (Higgins, Gebler & Frömter, 1975). Marked stimulation of net Na transport to levels observed in natural epithelia would not be anticipated in MDCK monolayers without an increased Na–K pump density. In low resistance/high potential urinary bladders, maintaining high short-circuit currents, a correlation exists with a high conductance of the basal lateral membrane; a result consistent with increased pump number (Frömter & Gebler, 1977). Aldosterone stimulation of the short-circuit current in TB–M and TB6C cell lines (Handler et al., 1979) may, therefore, be accompanied by increased Na-pump density. In other cultured cells such as HeLa cells there is evidence to suggest that increased levels of intracellular Na stimulate increased Na pump density (Boardman et al., 1974). The recent finding by Rabito and Tchao (1980) of changes in Na–K pump density in MDCK cells during monolayer organization and cell cycle may focus attention towards the regulatory possibilities of Na–K pump density upon transepithelial transport. In systems such as avian salt gland, increased cellular Na–K pump density is associated with increased cellular infoldings and membrane area (Ernst & Mills, 1977; Hossler, Sarras & Allen, 1978; Hossler, Sarras & Barnett, 1978).

It is of interest to observe that extensive amplication of basal-lateral membrane area as is observed in natural epithelia with high salt and water transporting capability (Schifferdecker & Frömter, 1978; Welling, Welling & Hill, 1978; Welling & Welling, 1979) is absent in all the established cell lines forming epithelial monolayers.

Finally, it is possible that there is variation in other MDCK cell functions dependent upon the existence of separate strains of MDCK cells. Rindler et al. (1979) report an adenyl cyclase responsive to anti-diuretic hormone and prostaglandins similar to high resistance distal/collecting tubules. No report has yet described a physiological correlate of this hormone stimulation of cAMP; I note that these agents

have no effect upon short-circuit current in low-resistance monolayers whereas a higher SCC is observed plus ADH and PGE_1 in the high-resistance layers (*unpublished observations*). The existence of separate strains of cells resulting in diverse cellular properties may also be relevant in the LLC Pk_1 cell line where, though a Na-dependent uptake system for sugars has been reported (Mullin, Weibel, Diamond & Kleinzeller, 1979), LLC Pk_1 epithelial monolayers do not display the characteristics expected of proximal tubule epithelia (Handler et al., 1980).

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