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IDENTIFICATION OF TWO STRAINS OF MDCK CELLS WHICH RESEMBLE SEPARATE NEPHRON TUBULE SEGMENTS

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Summary

Cultured monolayers of dog kidney (MDCK) cells display many features of in vivo epithelia. This work describes the identification of two separate strains of MDCK cell with entirely different properties.

Strain I cells form epithelial monolayers which display a high electrical resistance (4.1 $k\Omega \cdot cm^{-2}$); the basal short-circuit is small (approx. 0.5 $\mu amp \cdot cm^{-2}$) and is stimulated by adrenaline (1 μm) prostaglandin E_1 (1 μm) and arginine vasopressin (2 μm) added to the basal bathing solution. Strain II cells form epithelial monolayers of low electrical resistance; the short circuit current is insensitive to adrenaline, prostaglandin E_1 and vasopressin.

Strain II cells possess measurable activities of alkaline phosphatase and γ-glutamyl transpeptidase whereas Strain I cells do not. The specific activity of the $(Na^+ + K^+)$-ATPase is two-fold greater in Strain II compared with Strain I.

The polypeptide composition of the apical membrane differs substantially between the two cell strains as revealed by radio-iodination of external membrane proteins. Monolayer morphology is substantially different between the two cell strains.

The results are discussed in relation to previous work on MDCK epithelia, and the two types of cell monolayer are compared with in vivo tubule segments.

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Abbreviation: SDS, sodium dodecyl sulphate.

Introduction

Mammalian renal tubular epithelium consists of at least seven different segments and biochemical investigation of tubular tissue is complicated by this cellular heterogeneity. Partially purified populations of separate tubule segment cells have been achieved using density gradient centrifugation [1] and free-flow electrophoresis [2]. Alternatively micro-dissection of identified tubule segments has been combined with microanalytical techniques [3,4,5]. Recently an approach using established cultured cell lines derived from renal tissue has become popular [6,7,8,9]. Useful cell lines are epithelial, appear homogenous and retain certain differentiated features in culture. An example is the canine-derived cell line MDCK which forms cell monolayers with a characteristic polarised epithelial morphology [10,11] (brush-border, apical cell-cell junctions and lateral spaces). Biochemical polarity of the cellular plasma membranes is evident from the localization of the $(Na^+ + K^+)$-ATPase to the basal-lateral membrane using [^{3}H]ouabain autoradiography [12] and from the demonstration of the asymmetric composition of the apical and basal-lateral membranes using lactoperoxidase-mediated radioiodination of externally exposed membrane proteins [13]. Extensive retention of in vivo characteristics is inferred from the presence in MDCK cytosol of a specific binding protein for mineralocorticoids [14], also an adenylate cyclase responsive to renal hormones (adrenaline, vasopressin and prostaglandin E_1 has been demonstrated [7,8].

Growth of MDCK epithelial monolayers upon permeable substrates such as Millipore filters, allows measurement of electrophysiological parameters of the epithelial layer [10,11]. The low electrical resistance of the epithelial layer and its junctional parameters indicate that the MDCK epithelium resembles proximal tubular segments [9,10,11]. The pattern of hormonal stimulation of the adenylate cyclase, however, is not that expected of proximal segments but closely resembles that of distal/collecting tubule segments [7,8].

Recently we have defined two strains of MDCK cells which display entirely different electrophysiological properties [15]. This paper describes additional features of these two cell types. We observe that the cells of the two strains are morphologically and biochemically distinct and that there is a correlation between certain of the measured parameters which resembles that found for tubule segments in vivo.

Materials and Methods

Cell culture

MDCK renal epithelial cells were obtained at 60 serial passages (April 1978) and at 109 serial passages (November 1976) hereafter referred to as Strain I and Strain II, from Flow Laboratories, Irvine, Scotland. Both cell stocks had been obtained by Flow Laboratories from the American Type Culture Collection at slightly earlier serial passage (56 and 100). Chromosome analysis confirmed that two strains were of canine origin. The cultures were judged free of myoplasma contamination in the electron microscope. Gentamycin antibiotic was used for all growth media. The characteristics of the two strains described are stable for at least 20 serial passages. Cells were grown in 50-cm^2 Roux

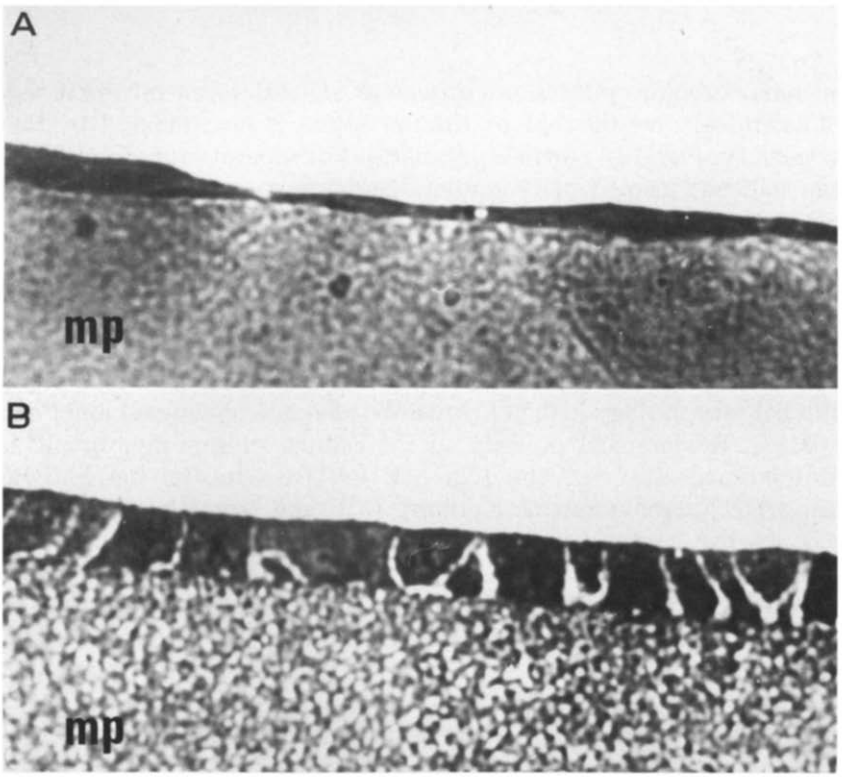


Fig. 1. Low power (X800) micrographs of semi-thin (0.5 μm) sections of Araldite-embedded epithelial monolayers of Strain I (A) and Strain II (B) cells. Stain was toluidene blue. mp, Millipore filter support.

bottles for enzyme studies as described in Ref. 7 or by seeding at high density [10] on Millipore filters of 2.5-cm diameter (0.22 μm pore diameter) for radioiodination and physiological determinations. Serum-free growth medium [9] was used for cell monolayers destined for radioiodination. Studies were made on confluent layers of cells after 5—7 days of growth. Confluent monolayers of cells were formed by both cell strains.

Resistance determinations alone are a good index of the formation of confluent monolayers in cell Strain I which form epithelial layers of high electrical resistance (see results). This was confirmed by fixation in glutaraldehyde and dehydrating selected cell monolayers, which were then examined using light microscopy, after rendering the Millipore filter transparent by toluene. For Strain II monolayers an additional criterion was used besides the resistance determinations. Replacement of the basal bathing solution NaCl by isosmotic replacement of mannitol, elicits a large potential difference. The basal bathing solution was electropositive, since the paracellular pathway in this cell type is cation selective [10,11,15]. The magnitude of this potential difference correlates with monolayer confluency as judged by light microscopy. Monolayers with dilution potentials $> +30$ mV are completely confluent. Holes in the monolayer result in the dilution potential approaching that expected of a free-solution pathway (—3—5 mV). Two low power views of confluent monolayers of Strain I and II cells are shown in Fig. 1.

Cell surface labelling and SDS-polyacrylamide gel electrophoresis

Lactoperoxidase-mediated iodination of apical surface polypeptides was performed as previously described [13] except that present results were obtained using cells grown in a serum-free medium and peroxide was generated in the iodination reaction by glucose oxidase [37]. To ensure specific labelling of the monolayer apical surface, monolayers were clipped into mini-Marbrook Chambers (Hendley Co.) thereby restricting access of experimental solutions to the apical surface. Cereijido et al. [11] have demonstrated the impermeability of MDCK tight junctions to peroxidase enzyme in cell monolayers of the low electrical resistance type (Strain II). A diffusion barrier to lactoperoxidase may also be inferred from the observed monolayer electrical resistance in situ for both epithelial strains (Table I). If a monolayer is damaged, this resistance is abolished (see also above).

Cell monolayers were rinsed in isotonic phosphate buffered saline (pH 7.4) for 30 min. Iodination was initiated by the addition of 0.25 cm^3 of iodination mixture (250 μCi $Na^{+125}I$/1.25 μg lactoperoxidase/0.125 μg glucose oxidase/ 0.25 μmol D-glucose in phosphate buffered saline); iodination was for 20 min at 37°C. Subsequent to iodination, monolayers were washed in ice-cold phosphate buffered saline for 15 min to remove unbound ^{125}I.

Analysis of radioiodinated polypeptides was made by electrophoresis in 16% SDS-polyacrylamide slab gels. Fixation, staining, molecular weight markers and autofluorography were as described in Richardson and Simmons [13].

Light and electron microscopy

Cell monolayers were fixed in 2% glutaraldehyde, post-fixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in Araldite. Semi-thin or thin sections were cut with glass knives. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philip EM301 at 80 kV. Semi-thin sections were stained with toluidine blue and examined using bright-field illumination.

TABLE I

THE ACTION OF HORMONES ION TRANSPORT IN MDCK MONOLAYER

Net ion transport is given as the short-circuit current measured 2—3 min after hormone addition. All hormone additions were made to the basal millipore bathing solution. All errors are ± S.E. Numbers in brackets represent the number of separate determinations.

Condition	Short circuit current $\mu A \cdot cm^{-2}$	
	Strain 1	Strain 11
Control	0.36 ± 0.10 (5)	0.86 ± 0.24 (3)
1 μM Adrenaline	38.10 ± 7.20 (5) *	0.84 ± 0.22 (3)
Control	0.28 ± 0.04 (5)	0.72 ± 0.25 (3)
1 μM Prostaglandin E_1	3.63 ± 0.64 (5) *	0.60 ± 0.10 (3)
Control	0.20 ± 0.04 (24)	0.60 ± 0.36 (3)
2 μM Arginine vasopressin	3.07 ± 0.63 (24) *	0.30 ± 0.26 (3)

* Significantly different from control values $P < 0.01$.

Enzyme determinations

Confluent Roux bottles were first washed in phosphate-buffered saline. The cells were freed from the glass substratum by scraping, centrifuged at 1000 × *g*, resuspended in 1 cm^3 Tris-HCl buffer (50 mM Tris-HCl pH 7.4) and finally homogenised. Homogenization was carried out at 0°C using 40 strokes of a glass Teflon homogenizer driven at 700 rev./min. Cell disruption was judged by light microscopy to be complete. Alkaline phosphatase activity was assayed as described by Berner and Kinne [16] using *p*-nitrophenyl phosphate as substrate. Aminopeptidase was determined using leucine *p*-nitroanilide as substrate [17]. γ-glutamyltranspeptidase was measured according to the method of Sigma (Bulletin 415) using L-γ-glutamyl-*p*-nitroanilide substrate. $(Na^+ + K^+)$-ATPase activity was measured by the decrease in absorbance at 340 nm during the transit of $NADH^-$ to NAD in the presence of phosphoenlolpyruvate, pyruvate kinase and lactate dehydrogenase [18]. Plasma membrane $(Na^+ + K^+)$-ATPase activity was estimated as being the activity remaining after subtraction of the ouabain insensitive component from the total. Ouabain, a known inhibitor of (Na^+, K^+)-pump function in MDCK cells [12,31,34], was used at 10^{-4} M. Enzyme activity remaining in the presence of ouabain is described as Mg^{2+}-ATPase. Protein was determined according to Lowry et al. [19].

[^{3}H]ouabain binding

[^{3}H]ouabain binding may be employed to measure the number of Na^+-pump sites. [^{3}H]Ouabain binding was determined in MDCK cells grown to sub-confluency upon plastic Petrie dishes as described by Boardman et al. [32]. (Ouabain, $5 \cdot 10^{-7}$ M; incubation time, 20 min [12].) Specific binding is the difference between binding in K^+-free and 14 mM K Krebs' solution. Sub-confluent monolayers were used to allow access to the basal surfaces. Confluent monolayers give similar levels of [^{3}H]ouabain binding indicating access to basal sites through collapsed or bursting blisters or at the edge of the plate. Cell numbers and volumes were measured directly by releasing cells using trypsin-EDTA and counting using a Coulter Counter [36].

Electrophysiological measurements

Cell monolayers were mounted in Ussing chambers (0.75 cm window radius, 1.76 cm^2 exposed monolayer), thermostated at 37°C similar to those used for rabbit small intestine [20]. An automatic voltage clamp [20] was connected to the Ussing chamber via matched calomel half cells (for potential measurement), Ag/AgCl half cells (for current passage) and saturated KCl-agar salt bridges. The use of saturated KCl minimised error due to liquid junction potentials. All potential differences 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-polarising current pulses across the cell monolayer except where current was caried. Constant current conditions were ensured by including a series 300 kΩ resistor. Current was measured by the potential drop across a precision 1 kΩ resistor placed in series. In most experiments the monolayers were voltage clamped to zero and the short-circuit current recorded continually on a Houston Instru-

ments chart recorder. The bathing solutions were a modified Krebs' solution containing 137 mM NaCl/5.4 mM KCl/2.8 mM $CaCl_2$/1.2 mM $MgSO_4$/0.3 mM NaH_2PO_4/0.4 mM KH_2PO_4/12 mM HCl/14 mM Tris-base/10 mM glucose/2 mM glutamine/2 mM sodium pyruvate/2% v/v foetal bovine serum/5 cm^3 Eagles medium amino acids (Flow Laboratories); pH = 7.4.

Chemicals

All reagents were of Analar grade. Enzymes, arginine vasopressin and adrenaline were obtained from the Sigma Chemical Company, Poole, Dorset. Prostaglandin E_1 was a gift from Dr. J. Pike of Upjohn Ltd., Kalamazoo, Michigan, U.S.A. and was added as an ethanolic solution.

Statistics

Variation in results is expressed as ±S.E. of the mean except where indicated. Significance of differences between mean values was tested using Student's *t*-test (two-tailed, for independent means).

Results

Growth of MDCK cells upon permeable Millipore filters results in the formation of confluent epithelial layers (Fig. 1) which, when mounted in Ussing chambers, possess both an electrical resistance (indicating diffusion limitation

TABLE II

CELLULAR DENSITY OF THE [Na^+,K^+)-PUMP

Figures in brackets represent the number of separate determinations. S.D. = ± standard deviation. All other errors are expressed ± S.E. The significance of the difference between strains is tested by Student's two-tailed *t*-test (unpaired means). Ouabain-sensitive ATP splitting is from paired determinations.

		Strain 1	Strain 11	Significance of difference
ATP splitting μmol min/mg cell protein	—Ouabain	0.119 ± 0.065 (3) S.D.	0.171 ± 0.014 (3) S.D.	n.s.
	+Ouabain	0.094 ± 0.071 (3) S.D.	0.114 ± 0.081 (3) S.D.	n.s.
	Ouabain sensitive component (a—b)	0.025 ± 0.010 (3) S.D.	0.053 ± 0.006 (3) S.D.	$P < 0.01$
[^{3}H]Ouabain binding	No K^+	2.23 ± 0.22 (5)	5.25 ± 0.13 (5)	$P < 0.01$
mol/cell · 10^5	15 mM K^+	0.57 ± 0.02 (5)	1.42 ± 0.17 (5)	$P < 0.05$
	Specific binding	1.65 ± 0.20 (5)	3.85 ± 0.28 (5)	$P < 0.01$
Estimate of number of cells/mg protein *		$1.3 \cdot 10^7$	$1.44 \cdot 10^7$	

* Determined in a separate experiment.

between the cells) and an active transport potential (indicating trans-epithelial ion transport). The resistance of Strain I is 4.16 ± 0.24 (n = 231) $k\Omega \cdot cm^{-2}$, similar only to mammalian distal/collecting tubules [21], that of Strain II is considerably lower, 71 ± 6 (n = 47) $\Omega\ cm^{-2}$, a value indicating a ('leaky') epithelium similar to proximal tubules [22].

Both cell strains possesss a basal-surface positive spontaneous potential difference that for Strain I is 2.17 ± 0.13 (n = 231) mV and for Strain II 0.17 ± 0.03 (n = 47) mV. The short-circuit current in both cell-strains is, therefore, small (Table 1) compared with natural epithelia. Addition of adrenaline, prostaglandin E_1 or arginine vasopressin to the basal bathing solutions of Strain I cell monolayers results in a stimulation of the short circuit current in the same sense as the spotaneous (basal) short circuit current. This result provides a physiological correlate to the known action of these hormones upon the adenyl

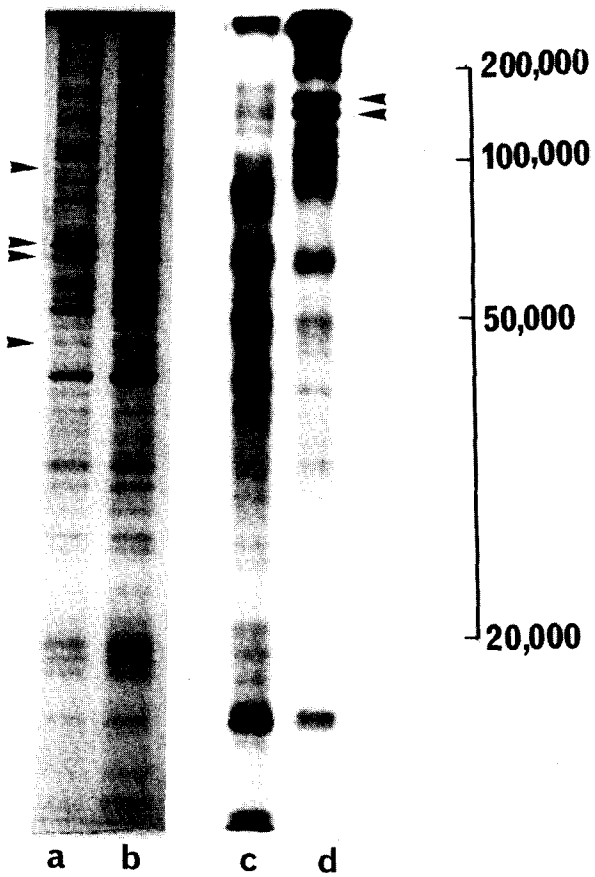
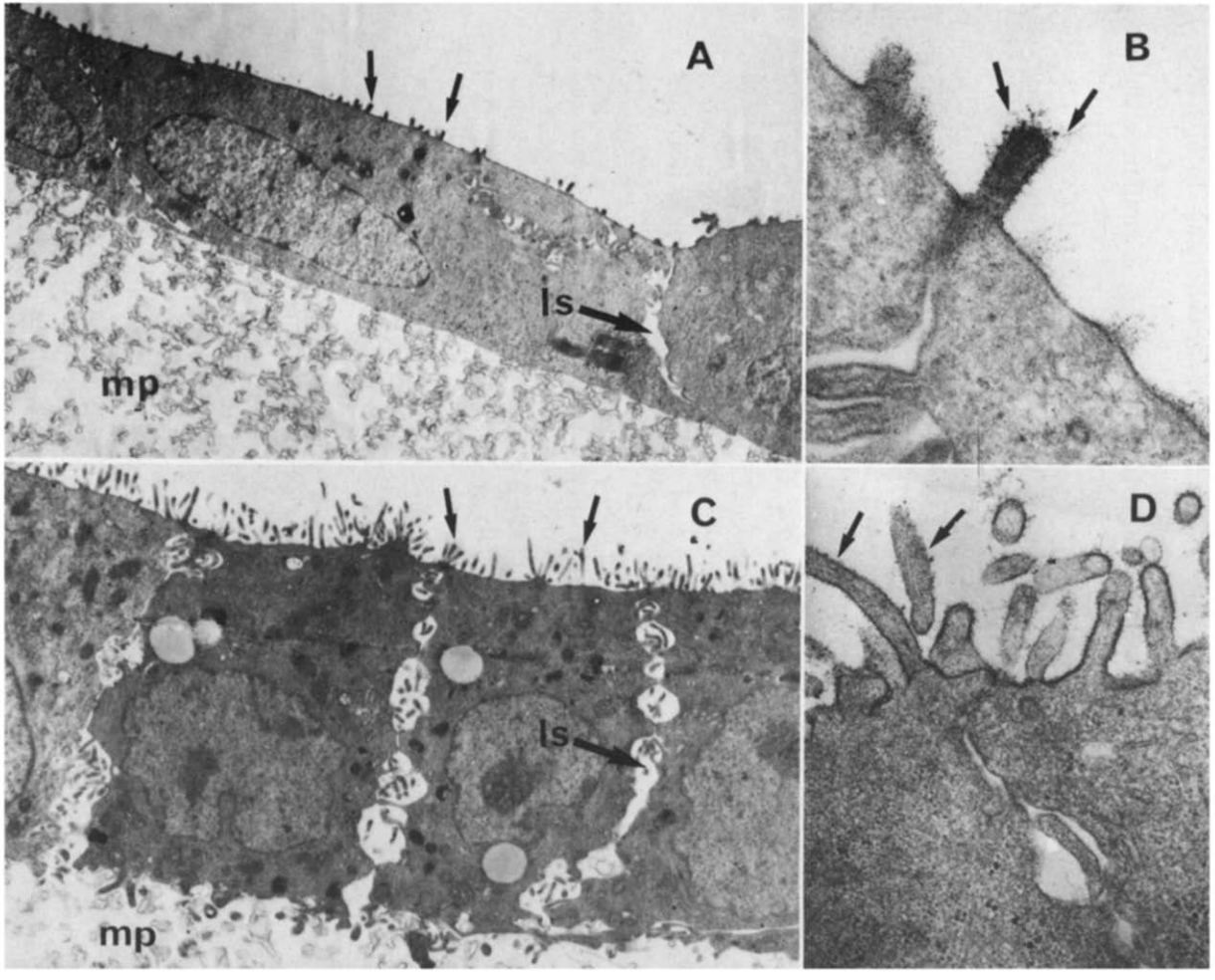


Fig. 2. Electrophoretograms of MDCK cell monolayers at similar protein and radioactive loading (a) Coomassie brilliant blue-stained electrophoretic pattern of whole cells (Strain II). (b) Equivalent whole-cell pattern from Strain I. Arrows denote homologous bands where quantitative differences in protein content exist. (c and d) Autoradiograph of apical iodinated externally exposed membrane proteins for Strain II and I respectively. Arrows denote the major apical protein doublet (160 000 and 130 000 apparent M_r) reported previously in Strain I [13], and which appears to be almost completely absent from Strain II.



Fi. 3. (A) Low power view (×8600) of a confluent epithelial layer of MDCK cells (Strain I) grown upon a Millipore filter (mp). Note the relatively flattened cell shape, the sparse brush border (arrowed) and the lateral cell space. (B) High power view (×91 000) of apical junctional area (Strain I). Note extensive staining of glycocalyx. (C) Low power view (×6250) of a confluent cell layer of Strain II MDCK cells. Note the columnar appearance of the cells, the extensive brush border (arrowed) and the wide lateral spaces (ld). (D) High power view (×45 000) of junctional area (strain II). Note the difference in microvillus size and staining compared to B.

cyclase [17,18] in this cell-line. The ionic basis to the increased short circuit current due to hormone stimulation determined by isotopic flux measurements is similar to that seen for purinergic stimulation [38] and forms the basis of a separate report. In Strain II no increased short circuit current is observed upon addition of either adrenaline, prostaglandin E_1 or arginine vasopressin. In rabbit microdissected tubule segments an adenylate cyclase responsive to vasopressin and adrenaline is found only in cortical collecting duct segments [4,5].

The specific activity of the $(Na^+ + K^+)$-ATPase is important since transepithelial Na^+ transport is dependent upon this enzyme and other solute transports are in turn linked to the Na^+ gradient [22]. The levels of $(Na^+ + K^+)$-ATPase are known to vary along the nephron tubule in various mammals [25]. In MDCK cells the specific activity of the ouabain-sensitive $(Na^+ + K^+)$-ATPase is two-fold greater in Strain II compared with Strain I (Table II). This is similar to the distribution of $(Na^+ + K^+)$-ATPase in vivo where proximal activity is greater than that of the collecting tubule [25]. The specific activity of the $(Na^+ + K^+)$-ATPase is of a similar order to that observed in homogenates of kidney cortex [17,18,26,36]. The Mg^{2+}-ATPase (ouabain-insensitive) is similar in both MDCK cell lines.

The difference in maximal specific activity of the $(Na^+ + K^+)$-ATPase is mirrored in the number of $(Na^+—K^+)$-pump sites, measured directly using [^{3}H]-ouabain binding (Table II), being more than two-fold greater in strain II cells. The level of specific [^{3}H]ouabain binding is of a similar order to cultured HeLa cells [31,32]. By using a conversion factor for number of cells per mg cell protein (Table II) it is possible to calculate a maximal turnover number for the $(Na^+—K^+)$-ATPase in MDCK cells (120 mol ATP split per s for Strain I cells and 95 for Strain II). Although the possible error for this type of calculation is quite large, it is nonetheless noteworthy that the values are similar in both cell types and within the range reported for the $(Na^+ + K^+)$-pump in various other cell types by Baker and Willis [31].

Alkaline phosphatase and γ-glutamyl transpeptidase are primarily localized in proximal segments [27]; these enzymes were detectable only in cells of Strain II (alkaline phosphatase activity = 35.2 ± 12.1 (n = 4) nmol/mg cell protein/min, γ-glutamyl transpeptidase activity = 20.8 ± 1.2 (n = 4) nmol/mg cell protein/min). In Strain I cells these enzyme activities were not detectable (the assay limits being <1 nmol/mg protein/min). The levels of activity of these enzymes in Strain II cells is, however, reduced as compared with kidney cortex homogenates [36].

The segmental distribution of leucine aminopeptidase is unknown, though this enzyme is often used as a brush-border marker enzyme for kidney epithelial cells; the specific activity of leucine aminopeptidase is similar in both MDCK cell strains (Strain I activity 9.6 ± 38 (n = 3); Strain II, 10.8 ± 2.4 (n = 3) nmol/mg cell protein/min) and is of a similar order to cortical homogenates [36].

The electropheretograms (Fig. 2) showing total cell protein of both MDCK cell strain (Coomassie brilliant-blue pattern) are very similar, though some quantitative differences in homologous bands do exist. In contrast, the patterns of iodination of externally exposed apical surface-membrane proteins from the two cell strains are substantially different. Homologies are of course present,

but the major protein doublet at apparent M_r of 160 000 and 130 000 in Strain I [13] is virtually absent from Strain II. In addition to these clear differences relating to the apical surface of the cells, there are striking ultrastructural differences between the apical surfaces of Strain I and Strain II cells (see below).

Fig. 3 shows typical sections of monolayers of each cell strain. Apical junctional complexes consisting of zonae occludentes (and desmosomes) exist between all cells. The distribution of cytoplasmic constituents is similar in both cell types and is similar to that described by Cereijido et al. [11]. There are, however, marked differences in cell size, Strain II cells being larger and taller ($8 \times 8 \times 10$ μm) compared with Strain I cells ($7 \times 7 \times 3.3$ μm). The distribution and size of the apical microvilli also differ substantially. Microvilli are short (0.2 μm) of low density ($5/\mu m^2$) and stain heavily with lead citrate and uranyl acetate in Strain I cells, whilst in Strain II cells the microvilli are longer (1—1.5 μm) of higher density ($25/\mu m^2$) and stain less heavily. These differences have now been seen in several separately cultured monolayers. Moreover, scanning electron microscopy has confirmed the differences in microvillus size and density (unpublished data). Similar differences in morphology are observed in renal tubules [23] and in other natural epithelia [24].

Discussion

This work demonstrates the existence of two separate strains of MDCK cells which exhibit quite distinct physiological, biochemical and morphological characteristics.

This finding has implications for previous studies in which the MDCK cell line has been used as an in vitro model of renal epithelial function. In particular the possibility of a heterogenous cell population exists, so reports of variation in junctional properties in MDCK layers [34] need to be interpreted with caution. Also reports concerning the action of dibutyryl cyclic nucleotides, derivatives and cell-transforming agents upon blister formation [28,29] and the nature of a defined serum-free growth media for MDCK cells and renal cells in culture [9] need now to be correlated with physiological or biochemical properties of the cell type used.

On the basis of their electrical resistance, their responsiveness to adrenaline and vasopressin and in the absence of proximal marker enzymes such as alkaline phosphatase and γ-glutamyl transferase, Strain I cells resemble collecting-duct segments. Strain II cells resemble proximal tubules since the short circuit current is not stimulated by adrenaline and vasopressin; these cell monolayers possess a low electrical resistance and the proximal tubule enzyme markers, alkaline phosphatase and γ-glutamyl transferase are both detectable.

It would be naive, however, the expect a complete co-identity with separate nephron segments since the MDCK cell line is established and adapted to culture conditions. Thus Strain II cells do not exhibit a Na^+-dependent sugar uptake system (unpublished data) and the levels of alkaline phosphatase are greatly reduced as compared with proximal segments. Also the magnitude of basal ion transport in both cell strains, as indicated by the short circuit current, is small compared to in vivo epithelia.

Although we have described two distinct MDCK cell strains it is possible that

others may exist. Strain II which has undergone a greater number of serial passages in culture may be a de-differentiated form of that present initially; in which case a range of properties between two extremes might be observed. Notwithstanding this possibility, the data reported here should extend the potential of MDCK epithelia as useful models of remal epithelial cell function.

Acknowledgments

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