Cyclic-AMP Deficient MDCK Cells Form Tubules

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Abstract   It has been known for many years that MDCK cells form blister-like structures, termed domes. During an examination of the morphology of a large number of MDCK clones, we found that two stable morphotypes exist in an MDCK cell population—namely, dome-forming and tubule-forming clones. When maintained at high cell density, tubule-forming clones displayed large numbers of anastomosing tubules which contained lumens. The frequency of observation of the tubule-forming clones in an MDCK population was 0.7%. Tubule-forming MDCK clones should be useful in studying tubule morphogenesis. While agents that affect protein kinase A activity increased dome formation, the same agents abolished the formation of tubules in all tubule-forming clones. In contrast, drugs that stimulate protein kinase C activity (phorbol esters and staurosporine) decreased dome formation and increased tubule morphogenesis in all MDCK morphotypes. Tubule-forming clones were found to have lower resting levels of cyclic-AMP and to respond to forskolin stimulation of adenylate cyclase less readily. Hence, signals transmitted by the protein kinase C pathway appear to lead to tubule formation in MDCK cells, while signals transmitted through the protein kinase A pathway lead to dome formation.   © 1995 Wiley-Liss, Inc.

Key words: cyclic-AMP, morphogenesis, MDCK cells, protein kinase A, protein kinase C, signal transduction

In 1969, Leighton and his colleagues demonstrated that Madin-Darby canine kidney (MDCK) cells form fluid filled, blister-like structures, termed domes [Leighton et al., 1969]. Domes form due to a unidirectional flow of salt into a lumen formed between a solid substratum and the basolateral cell surface. Following secretion of ions into the lumen [Abaza et al., 1974; Grant et al., 1991], osmotic pressure results in the flow of water into the lumen with the resulting inflation of a blister-like dome. Dome formation is inhibitable by treatment of cells with ouabain, an inhibitor of the cell sodium-potassium pump [Abaza et al., 1974; Cohen-Luria et al., 1993]. It is known that the frequency of dome formation can be increased by DMSO and cyclic-AMP [Lever, 1979; Thomas et al., 1982], agents that induce cell differentiation in other cell systems [Reuben et al., 1980; Klebe and Mancuso, 1982, 1983].

We began studies of the MDCK cell line due to our interest in the morphogenesis of cells maintained at tissue-like cell densities [Klebe et al., 1991]. Since structures, such as domes, are eas-

ily quantifiable, we were interested in examining MDCK as a model system for studying morphogenesis in vitro. Examination of clonal derivatives of MDCK revealed the presence of rare tubule-forming clones that had a markedly different morphology than the mass culture from which they were derived. In this report, we describe the morphological characteristics of tubule-forming derivatives of MDCK and factors that promote alternative patterns of morphogenesis in this system.

MATERIALS AND METHODS
Cyclic-AMP Assays

Cyclic-AMP was assayed with a TRK432 Cyclic AMP [3H] assay kit (Amersham Corp., Arlington Heights, IL) as described by the manufacturer. In brief, 10^6 cells (see text) were planted in 5 ml of complete culture medium in 60 mm cell culture plates and treated with drugs after an overnight incubation. Cyclic-AMP was extracted from cells by treatment of cells for 10 min with 2:1 ethanol:water. Following removal of the 2:1 ethanol-water under high vacuum in a SpeedVac (Savant Instruments, Framingdale, NY), cyclic-AMP was assayed according to the manufacturer.

Cell Layer Resistance

The resistance of the cell layer was measured by the method of Giaever and Keese [1991] using an Electrical Cell-Substrate Impedance Sensor (ECIS) system supplied by Applied Bio-Physics, Inc. (Troy, NY). The cells under study were cultured on small gold electrode (area 5.6×10^{-4} cm^2) affixed to the surface of a well of a 96-well plate bathed in tissue culture medium which also acted as the electrolyte. The electrodes were connected to a dual-phase lock-in amplifier (Stanford Research Systems model SR830, Sunnyvale, CA) interfaced with a personal computer which was used to both control the operation of the lock-in amplifier as well as to record the data collected by it. Cells were seeded at a density of 5×10^4 cells/well, and thereafter the changes in resistance of the electrode were recorded at regular intervals at a frequency of 4,000 Hz. The resistance measured has been shown to represent the transepithelial resistance [Giaever and Keese, 1991].

Cell Culture

MDCK cells (ATCC CCL 34) were obtained from the American Type Culture Collection (Rockville, MD). The MDCK variant clones, SuperTube and SuperDome, have been submitted to the American Type Culture Collection for distribution. Cells were maintained in 50% Dulbecco's MEM/50% Ham's F-12 containing 10% newborn calf serum plus 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin sulfate. Cells were maintained in log phase, and subconfluent cultures were fed the night before an experiment.

MDCK cells were cloned in 96-well plates which contained 0.25 ml of medium/well. Clones were given a complete medium change at days 7, 14, 18, 20, and 22. Once clones had grown to confluence and displayed their morphological phenotype (19–21 days), clones were scored for their ability to form either domes or tubules.

Quantitative Analysis of Morphogenesis

In several prior studies, dome formation has been followed by simply counting the number of domes/microscopic field and/or measuring the area of domes [Lever, 1979; Taub et al., 1989]. The following technical modifications of earlier methods were employed to expedite the quantitation of MDCK cell morphogenesis. Briefly, MDCK cells were plated at 7×10^5 MDCK

cells/well in a 24-well plate (Corning Glass Works, Corning, NY) in 2.5 ml of medium. Each well was given a complete medium change at 24 h intervals. By planting MDCK cells at very high cell density at t_0, we could detect dome formation within 24 h rather than after many days as in prior studies [Lever, 1979; Taub et al., 1989]. In the experiments reported here, cultures were fixed after 48 h in neutral buffered formalin and stained with Wright's stain (Sigma, St. Louis, MO). To count domes, a camera lucida device (Nikon, Japan) was used to project enlarged images of microscope fields directly from the microscope. Domes were counted by placing a plastic film over the camera lucida screen and marking domes with a marker pen whose ink could be removed with alcohol. Use of the camera lucida device eliminated time-consuming photography and printing. Dome area was determined by weighing the image of domes cut from enlarged photographic prints. Tubule length was determined with a map reading device (Keufells-Esser, Switzerland) normally used for determining distances on maps. Map units were converted to microns with the aid of a stage micrometer.

Time-Lapse Cinemicrography

Time-lapse studies were performed with a special chamber designed to permit observation of cells growing at very high cell density [Klebe, Grant, and Grant, 1993]. In brief, the chamber consists of a 25 cm^2 flask equipped with inlet and outlet ports that permitted the flask to be perfused with medium at desired intervals. A low light video surveillance camera (RCA model TC2511U8) was used to obtain images that were recorded by a Panasonic time lapse video tape recorder (model AG-6030).

Histology

Cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylic acid, pH 7.2, and then prepared for TEM or SEM observation. For scanning electron microscopy, ligand-mediated osmium binding [Kelley et al., 1973] was employed due to the fragility of the specimens. TEM specimens were postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol/water solutions, and embedded in PolyBed 812 resin (Polysciences, Warrington, PA). Once hardened, the embedded cells were separated from the plastic culture surface and treated at 80°C overnight to complete the polymerization process.

Sections were cut on a Sorvall MT-5000 Ultramicrotome at 800 Å and placed on formvar/carbon coated 200 mesh grids. Sections were stained with 7% aqueous uranyl acetate and Reynolds lead acetate. Specimens were examined with a Philips 301 electron microscope.

Reagents

Reagents were obtained from Sigma Chemical Co. Phorbol 12-myristate 13-acetate (PMA) was dissolved at 1 mg/ml in absolute ethanol. Matrigel was obtained from Collaborative Biomedical Products (Bedford, MA). Type I collagen was prepared from rat tail tendons [Klebe, 1974]. All other chemicals were of reagent grade.

RESULTS
Isolation of Tubule-Forming Variants of MDCK Cells

During the observation of MDCK clones which had grown to very high cell densities, we noted that a few rare clones produced large numbers of tubule-like structures (Fig. 1). Several tubule-forming clones were isolated and analyzed as described in the next section. That tubule-forming MDCK clones have not been observed in the past is probably due to the fact that MDCK clones were not allowed to reach extremely high densities in the past. Thus, by plating cells at densities comparable to very high density cultures, we were able to isolate clones whose phenotype would not be apparent at even moderately high cell density.

Four tubule-formers were isolated from MDCK (Fig. 1) and are termed Tube-1, etc. In addition, several extreme examples of dome-formers were also observed (Fig. 1), and clones, termed Dome-2 and SuperDome, were studied further. While MDCK domes have an average area of $3,384 \pm 303$ μ^2, Dome-2 forms large numbers of very small domes (average area = 803 ± 93 μ^2), while SuperDome forms
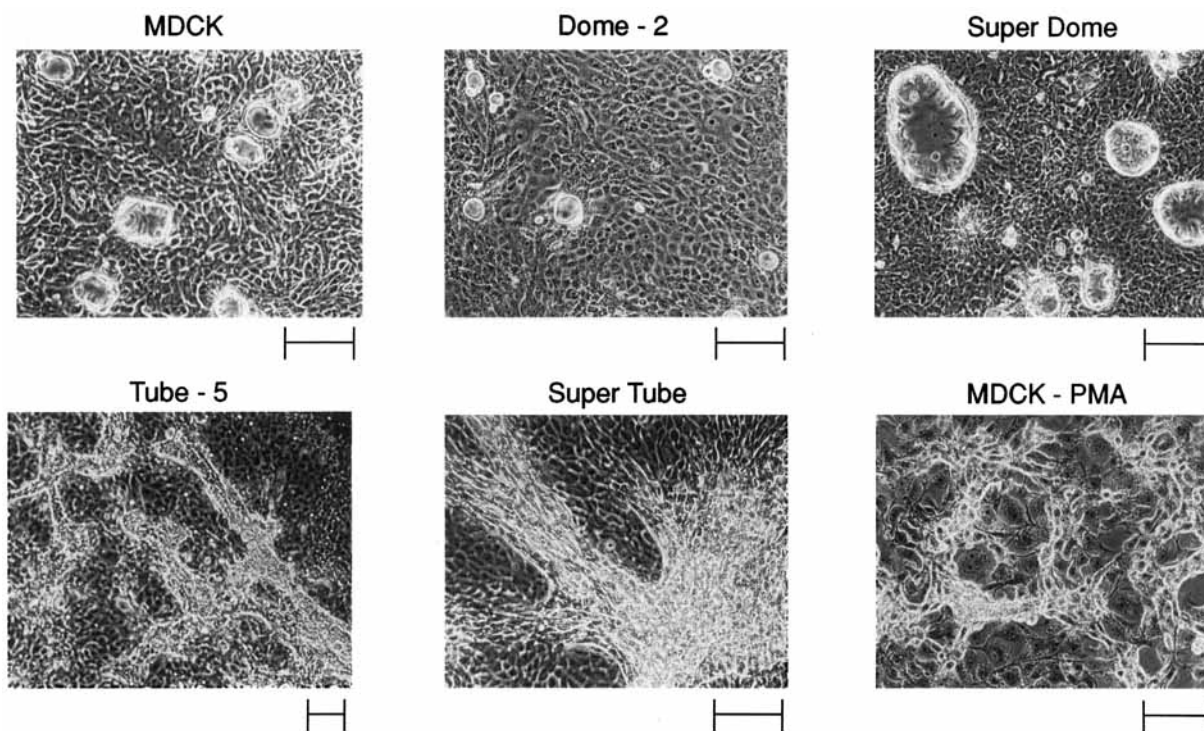

Fig. 1. Morphology of MDCK morphotypes. The morphology of the several representative MDCK morphotypes is presented. Cells were plated at 7×10^5 cells/well of a 24-well plate. Cultures were photographed with phase contrast optics using a 20× objective. The dome-formers (MDCK, Dome-2, and Super-Dome) produced domes of different sizes. While MDCK produced domes of 3384 μ^2 average area, Dome-2 produced large numbers of domes that were smaller (803 $\pm$ 93 μ^2 average area) than those of MDCK, while SuperDome displayed very large domes (4,751 $\pm$ 629 μ^2 average area). Large numbers of anastomosing tubules were formed by Tube-2 and SuperTube. MDCK forms partial tubules when treated with the phorbol ester, PMA. The bar represents 50 μ. While dome formation could be observed within 24 h, tubule formation required 2–3 days. By day 1, cells of tubule-forming clones exhibited arrays of parallel aligned cells that formed ridge-like structures. While domes form as cells lift from the culture surface as liquid is forced between the cells and the substratum, tubules have lumens surrounded on all sides by cells (see Figs. 2, 3).

very large domes (average area = 4,751 ± 629 μ^2).

Morphology of Tubule-Forming MDCK Clones

Scanning electron microscopy of a tubule-forming clone demonstrated the presence of anastomosing tubules (Fig. 2A). Due to the fortuitous fracture of some tubules in the specimen, it was possible to demonstrate that the tubules contained a lumen (Fig. 2B). Transmission electron microscopy also demonstrated the presence of lumens in multilayered cultures of tubule-forming clones (Fig. 3). Time-lapse cinemicrography of tubule-forming clones indicated that tubule formation was preceded by the parallel alignment of cells.
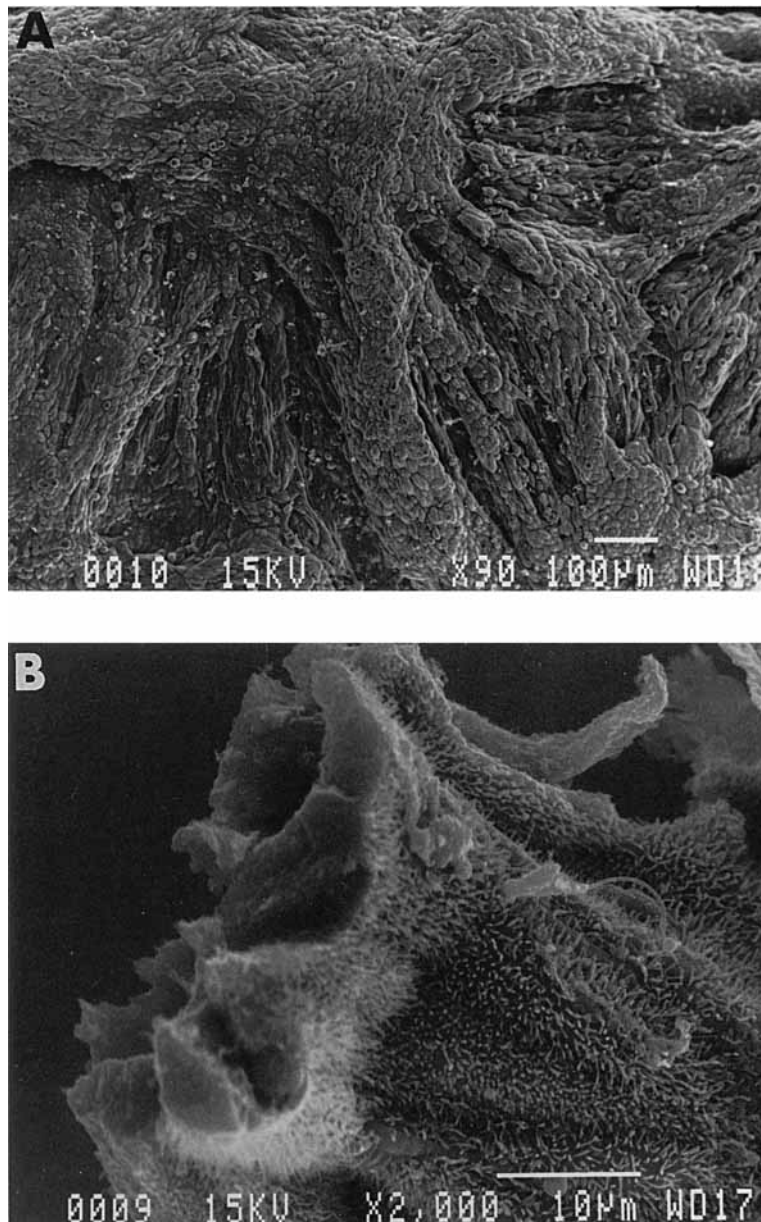

Fig. 2. Scanning electron microscopy of a MDCK tubule forming clone. SuperTube cells were planted at 7×10^5 cell well in a 24-well plate and fed each day until fixation at day 4. Cells were prepared for SEM as described in Materials and Methods. **A:** SEM of tubules formed by SuperTube; note the anastomosing tubules present in the specimen. **B:** The fortuitous fracture of a tubule demonstrates that tubules possess lumens. Optical sectioning, carried out by confocal microscopy of eosin stained cultures, has also demonstrated anastomosing lumens (data not shown).
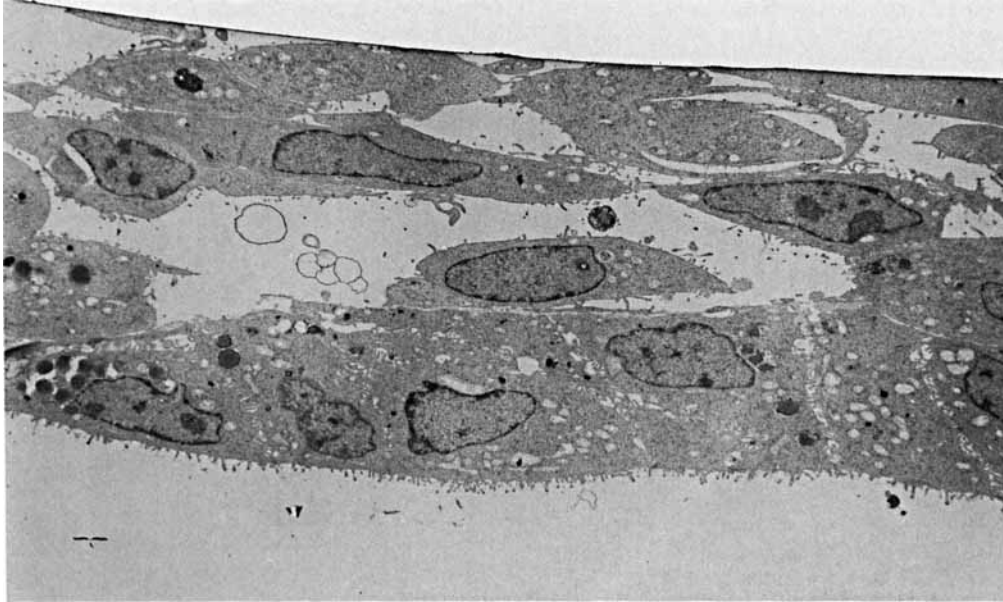
Fig. 3. Transmission electron microscopy of a tubule forming MDCK clone. TEM was used to confirm the presence of lumens in tubules as was demonstrated by SEM in Fig. 2. Cells were fixed as a monolayer and sectioned from the side to reveal a lumen in the tubules. The bar represents 0.7 nm.

Stability of MDCK Morphotypes

The parental MDCK cell line and several dome- and tubule-forming clones were subcloned to determine the stability of their morphological characteristics (Table I). Once subclones had attained tight confluence after approximately 21 days, each clone was examined and its morphotype scored. While most clones displayed either numerous domes or multiple anastomosing tubules, any clone displaying even one dome or tubule was scored as a dome-former or tubule-former, respectively. No clones were found to produce both domes and tubules. It should be noted that several subclones could not be categorized as either dome- or tubule-formers; many of these clones probably would have displayed their phenotype if cultured beyond 21 days.

The vast majority of subclones isolated were morphologically identical to the clone from which they were derived; however, with the exception of SuperDome that bred true, each morphotype spawned subclones of the opposite morphotype (Table I). Tubule-forming clones produced dome-forming subclones at a rate of 1.4–5.4%; it should be noted that such dome-forming subclones of a tubule-former displayed only one or a very few domes/well of a 96-well plate. MDCK produced tubule-forming clones at a rate of 0.7%, while its subclone, SuperDome, did not produce any tu-

bule-formers out of 223 subclones examined. Dome-2, which produces large numbers of very small domes, yielded tubule-formers at a rate of 14.8%.

Karotypic Analysis

In order to verify the MDCK origin of the tubule-forming clones, SuperTube and Tube-3 were karyotyped. The modal chromosome number of SuperTube and Tube-3 was 75 and 79, respectively, which is in agreement with the published value of 78 for MDCK [Hay et al., 1992]. In addition, two large metacentrics were noted in the tubule-forming clones that were observed in MDCK. Thus, the karyology of the tubule-forming clones indicates that they arose from MDCK.

Effects of Agents That Alter Signal Transduction Pathways on MDCK Morphogenesis

Since several drugs that affect signal transduction through protein kinase A have been shown to affect dome formation [Lever, 1979; Thomas et al., 1982], we examined the effects of agents that alter either protein kinase A or protein kinase C activity on tubule formation (Fig. 4). While we confirmed that a protein kinase A stimulator (forskolin) enhances dome formation (Fig. 4E), we found that the same protein kinase

TABLE I. Stability of Dome- and Tubule-Forming Variants of MDCK Cells*

Morphotype	Dome-forming clones (%)	Tubule-forming clones (%)	Neither dome- nor tubule-forming clones (%)
Dome-forming clones			
MDCK	85.5	0.7	13.6
Dome-2	56.1	14.8	29.1
SuperDome	93.7	0	6.3
Tubule-forming clones			
SuperTube	4.0	59.9	36.0
Tube-3	5.4	55.5	39.0
Tube-4	1.4	52.8	45.7
Tube-5	2.6	53.7	43.6

*Representative clones of each morphotype were recloned, and the frequency of occurrence of dome-formers and tubule-formers was tallied. The results indicate that dome-forming clones produce predominantly dome-forming subclones, while tubule-formers produce mainly tubule-forming subclones. MDCK, a dome-former, produced tubule-formers at a rate of 0.7%, while SuperDome did not produce a single tubule-forming subclone out of 223 subclones examined. Dome-2, which produces extremely large numbers of very small domes, produced tubule-forming subclones at a rate of 14.8%. All four of the tubule-forming clones examined produced dome-forming clones at a rate of 5.4% or less; note that such clones displaying domes had one or a very few domes/well. The analysis was carried out as follows. Cells were planted at clonal densities in 96-well plates, and the resulting clones were allowed to grow for 19–21 days such that each clone reached tight confluence. This period of time is required for a single cell to form a confluent culture for the following reason. We estimate that a confluent 96-well plate contains 4×10^4 cells. If one assumes a 24 h doubling time, more than 15 days of logarithemtic growth would be required for a single cell to produce a confluent culture. We noted that if the original cell attached near the walls of a well that even more time was required for confluence to be attained. The 96-well plates were fed on days 7, 14, 18, 20, and 22. Clones were scored as either a dome-former or a tubule-former if one or more domes or tubules were found in a well. In most cases, large numbers of domes or tubules were found in a single well. Clones were placed in the "neither dome- nor tubule-forming clone" category if neither a dome or a tubule was found in a well. Since very high cell density is required for the appearance of domes and tubules, clones in the "neither dome- nor tubule-forming clone" category probably represent clones that would form morphological structures if given additional time to mature. Alternatively, such clones may represent a third MDCK morphotype. Two hundred or more clones of each cell line were individually examined. The results presented were obtained from three separate experiments.

A stimulators prohibit the formation of tubules (Fig. 4F). During studies involving the fraction of conditioned medium with ammonium sulfate, we found that ammonium ion alone was a potent stimulator of dome formation (Fig. 4A). As in the case of forskolin, ammonium ion was found to suppress tubule formation (Fig. 4B). Ammonium ion has been shown to affect cell polarization in MDCK cells [Uchida et al., 1991]. The phorbol ester, PMA, which acts on protein kinase C [Woodgett et al., 1993; Anderson et al., 1985], was found to induce tubule morphogenesis in tubule- as well as dome-forming cells (Figs. 1, 4C,D). It was also found that another agent that stimulates protein kinase C, 0.1 μM Staurosporine, also induces tubule formation and inhibits dome formation (data not shown). Thus, dome-forming cells have the potential to form tubules following PMA stimulation which may indicate that signal transduction through the protein kinase C pathway is involved.

Measurement of cyclic-AMP levels indicated that the dome-forming cell lines, MDCK and SuperDome, had similar cyclic-AMP values/cell, while SuperTube cells had lower resting levels of cyclic-AMP. Upon stimulation of adenylate cyclase with forskolin, the dome-forming lines also displayed much larger increases in cyclic-AMP than SuperTube (Fig. 5). Since ammonium ion was similar to forskolin in promoting dome formation and inhibiting tubule morphogenesis (Fig. 4), we examined the effect of ammonium ion on cyclic-AMP levels. Between 1 min and 24 h, we found no effect of 10 mM ammonium chloride on cyclic-AMP levels in MDCK cells (data not shown). Thus, ammonium ion may alter morphogenesis in MDCK cells by a different mechanism.

Transepitelial Resistance Measurements

Electrical measurements were performed from the time that cells were planted for up to 60 h (Fig. 6). While both MDCK and SuperDome cells display an increasing transepitelial resistance during their first day after planting, SuperTube cells never approach the transepitelial resistance values recorded for their dome-forming counterparts.

Using calculations outlined by Giaever and Keese [1991], it can be shown that the transepithelial resistance of the MDCK and the SuperDome cells reach a value of ~ 100 Ω-cm^2 after 60 h, whereas that of the SuperTube cells is approximately 1 Ω-cm^2. It has previously been shown that MDCK has a transepithelial resistance of 100 Ω-cm^2, and a variant of MDCK has been reported with a transepithelial resistance of
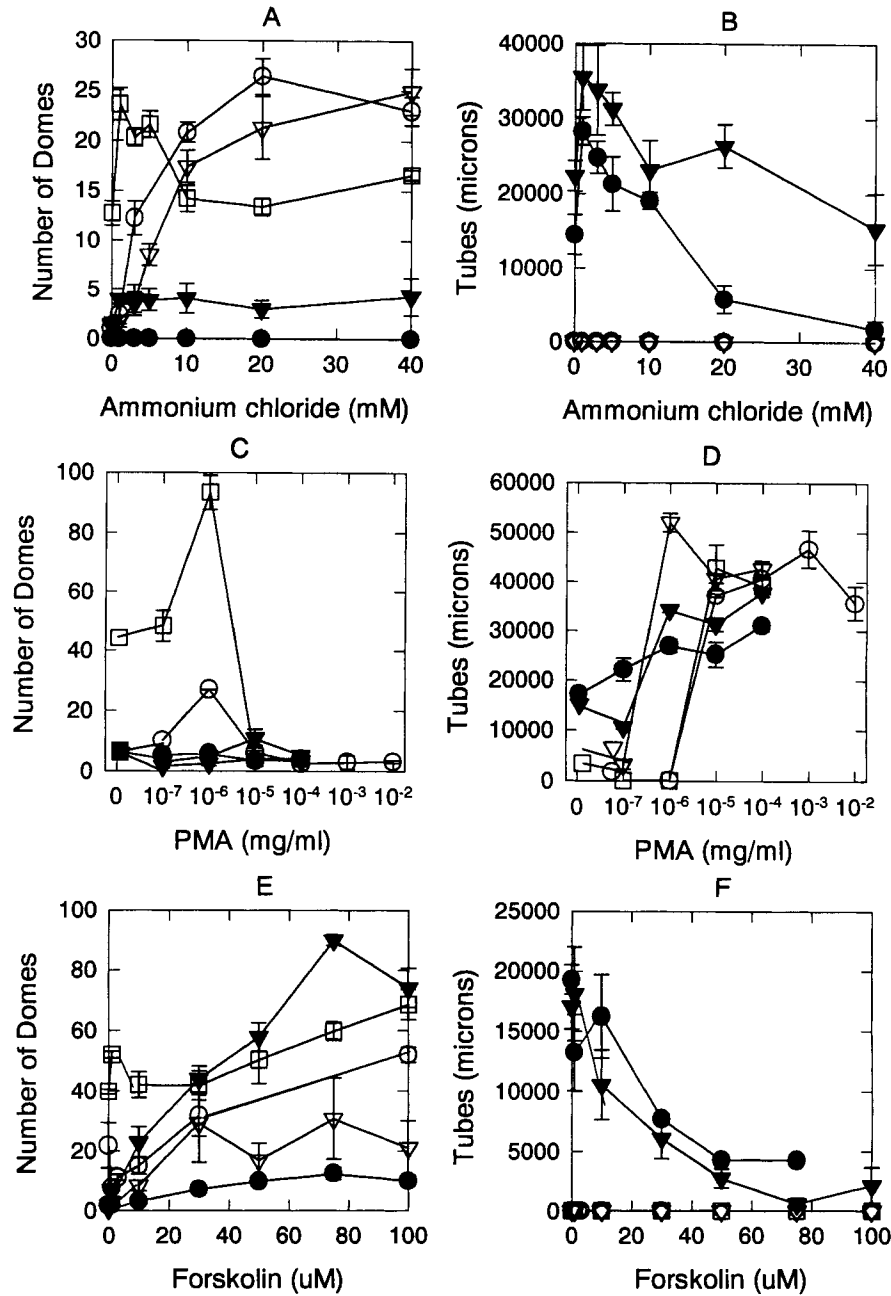
Fig. 4. A–F Effect of agents that alter signal transduction pathways on MDCK morphogenesis. Cells were exposed to drugs at the concentrations indicated, and morphological parameters (number of domes or length of tubules) were determined at each drug concentration. The techniques employed are described in Materials and Methods. Each data point represents the mean ± standard deviation of dome number or tubule length in ten or three microscopic fields (40× total magnifica-

tion), respectively. Note that solid symbols represent tubule-forming clones, while opened symbols represent dome-forming clones. Closed circle = Tube-3; closed triangle = SuperTube; opened circle = MDCK; opened triangle = Dome-2; opened square = SuperDome. The results indicate that forskolin and ammonium chloride stimulate dome formation and inhibit tubule formation; in contrast, PMA promotes tubule morphogenesis and inhibits dome formation.

4,000 Ω-cm^2 [Barker et al., 1981]. Thus, the electrical properties of SuperTube indicate that it is a new derivative of MDCK.

DISCUSSION

The Madin-Darby canine kidney cell line (MDCK) has been extensively studied since this
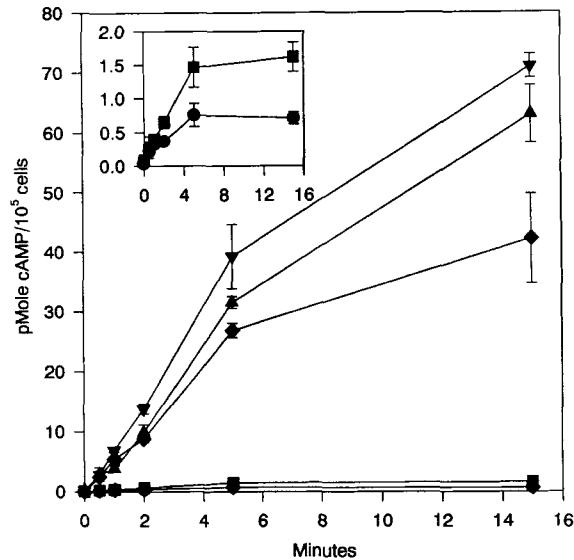
Fig. 5. Cyclic-AMP measurements. The resting cyclic-AMP levels of dome-forming cells (MDCK, SuperDome, and Dome-3) were found to be 0.28, 0.23, and 0.18 pMole/10^5 cells, respectively, while tubule-forming clones (SuperTube and Tube-3) had resting levels of 0.04 and 0.09 pMole/10^5 cells, respectively. Thus, all three dome-forming cell lines have higher cyclic-AMP resting levels than both tubule-forming clones. Following stimulation with 100 μM forskolin, the dome-forming cell lines displayed over a 200-fold increase in cyclic-AMP levels within 15 min, while SuperTube made only an eighteen fold response. Inset: Since the large response of dome-forming cell lines to forskolin made it difficult to display the tubule-forming clone results, the inset presents the tubule forming clones results using an expanded scale for the y-axis. Closed triangles = MDCK; closed inverted triangles = SuperDome; closed diamonds = Dome-3; closed circles = SuperTube; closed squares = Tube-3. Each data point represents the mean ± standard deviation of four determinations.

cell line can polarize components of its plasma membrane [Parton et al., 1991; LeBivic et al., 1990; Wang et al., 1990a,b; Kelley et al., 1973] and display transepithelial resistance [Thomas et al., 1982; Misfeldt et al., 1976]. MDCK cells cultured on Millipore filters mounted between two chambers display unidirectional fluid transport and ionic permeabilities similar to normal epithelia [Ojakian, 1981]. Thus, MDCK cells have become a model for many epithelial cell systems.

In this study, we have demonstrated that clones of MDCK cells arise at a frequency of 0.7% that are capable of undergoing morphogenesis into tubules rather than domes (Table I). In the early literature on MDCK cells, it was noted that "cordlike" structures are occasionally observed in confluent MDCK cultures [Abaza et

al., 1974]. Such cordlike structures probably were produced by clonal populations of tubule-forming cells, such as those described here.

That the morphotype of a clone is an inherited property rather than an effect of the environment is indicated by the finding that the morphotype of a given clone is inherited by its subclones (Table I). For example, 85.5% and 93.7%, respectively, of the progeny of both MDCK and Dome-6 are dome-formers, and greater than 50% of the clones derived from a tubule-forming clone are tubule-formers. As discussed in Table I, the above data probably underestimate the phenotypic stability of the cell lines studied. Hence, the morphotype of dome-formers and tubule-formers appears to be a stable trait. Nevertheless, the frequency at which conversion from one morphotype to another is observed is far higher than one would expect if a genetic mutation were involved.

Three possible mechanisms may account for the high rate of conversion of morphotypes observed in this system. First, MDCK may be a stem cell that can generate more highly differentiated stem cell lines. For example, the $10T_{1/2}$ cell line can generate clones which are committed to the myoblast, chondrocyte, adipocytes, and other pathways of cell differentiation [Lassar et al., 1986; Wright et al., 1989; Steffensen et al., 1992]. Second, errors in segregation of chromosomes to daughter cells in permanent cell lines occur frequently and can result in inheritable differences between clonal derivatives. In the case of MDCK, only 28% of cells display the modal chromosome number of 78, while cells are detected that have from 65–89 chromosomes [Hay et al., 1992]. Clones derived from MDCK (and most other permanent cell lines) are genetically heterogeneous due to derivation of clones from cells bearing different numbers of chromosomes or different combinations of individual chromosomes. Third, the tubule-forming clones may be altered in one or more elements of the protein kinase A or C pathways.

Any of the above models may be correct, and each model presented could result in changes in cyclic-AMP levels in tubule-forming clones. That cyclic-AMP levels are much lower in SuperTube than in dome-forming MDCK cells (Fig. 5) indicates that alteration in adenylate cyclase activity may control alternative pathways of morphogenesis in MDCK cells.
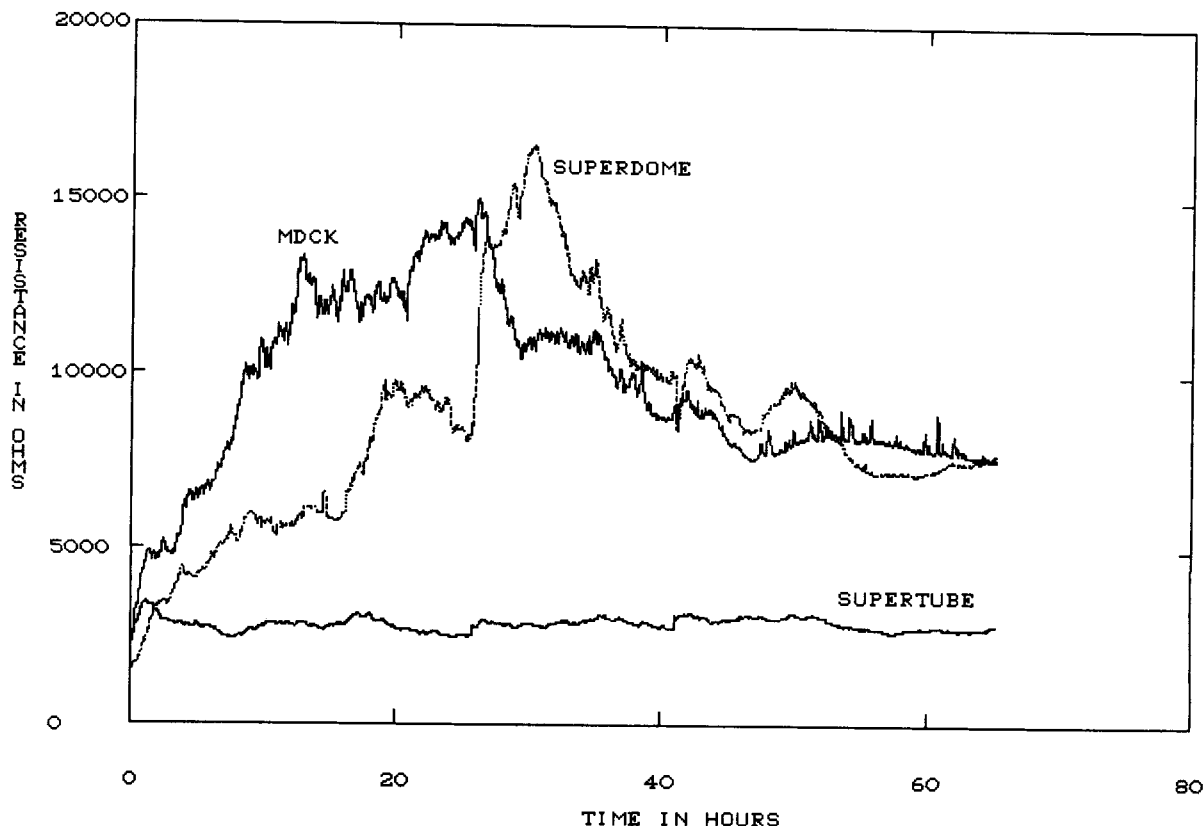
Fig. 6. Electrical measurements. When fully spread, MDCK and SuperDome cells are large and cover much more of the electrode area than the elongated SuperTube cells. This is reflected in the resistance change observed. The resistance change in the case of the SuperDome and the MDCK cells increases to approximately 20 times the original resistance. On the other hand, the transepithelial resistance for the SuperTube cells increases by only about threefold. After initial attachment and spreading, the measured resistance reflects the transepithelial resistance of the cell layer [Giaever and Keese, 1986, 1991]. The low resistance observed with SuperTube cells indicates that SuperTube cells have open spaces between them, whereas SuperDome and MDCK cells do not.

The following evidence provides additional support for the conclusion that signal transduction pathways control alternative patterns of morphogenesis in MDCK cells. Treatment of dome- and tubule-forming clones with agents that alter the protein kinase A and C signal transduction pathways was found to alter the expression of both morphotypes (Fig. 4). For example, dome-forming clones were found to produce tubules when treated with the protein kinase C stimulators, PMA, and staurosporine; in contrast, tubule-forming clones generated domes when exposed to the protein kinase A stimulator, forskolin (Fig. 4). These findings indicate that clones of either morphotype have the potential of generating the opposite morphotype (Fig. 4). Thus, morphogenesis of MDCK cells can take place under more than one developmental program, and program choice appears to be determined by crosstalk [Houslay, 1991]

between elements of the protein kinase A and C signal transduction pathways.

ACKNOWLEDGMENTS

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