

RETENTION OF DIFFERENTIATED PROPERTIES IN AN ESTABLISHED DOG KIDNEY EPITHELIAL CELL LINE (MDCK)

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ABSTRACT

Madin-Darby canine kidney (MDCK) cells grown in tissue culture have the morphological properties of distal tubular epithelial cells, form tight junctions, and lack several proximal tubular enzyme markers. Adenylate cyclase in these cells was stimulated by vasopressin, oxytocin, prostaglandins E₁ and E₂, glucagon, and cholera toxin. Hormone-stimulated adenylate cyclase activity in isolated membrane preparations was dependent on low concentrations of GTP and had the MgCl₂ and pH optima expected for the kidney enzyme. The results, as well as the demonstration of enhanced hemicyst formation induced by cyclic AMP, suggest that the MDCK cell line has retained the differentiated properties of the kidney epithelial cell of origin.

When MDCK cells were injected into baby nude mice, continuous nodule growth was observed until adulthood was attained. Histological studies revealed the presence of two cell types: normal mouse fibroblasts which comprised 80–90% of the solid nodule mass, and MDCK cells, which formed epithelial sheets lining internal fluid-filled glands. Electron microscope analysis showed that the mucosal surfaces of the cells were characterized by microvilli which faced the lumen of the glands, that adjacent MDCK cells were joined by tight junctions, and that the serosal surfaces of the epithelial sheets were characterized by smooth plasma membranes which were lined by a continuous basement membrane. These observations lead to the conclusion that the MDCK cells retain regional differentiation of their plasma membranes and the ability to regenerate kidney tubule-like structures *in vivo*.

KEY WORDS kidney epithelial cells · epithelial cell polarization · vasopressin · prostaglandins E₁ and E₂ · cyclic AMP · MDCK

Processes of central importance to the renal physiologist include: (a) the biogenesis of functional kidney tubules, (b) transepithelial salt and fluid

transport, and (c) hormonal regulation of transport functions (10, 34). Attempts to gain information about these processes have been hampered by the cellular complexity of the kidney. In general, it has not been possible to assign specific functions to individual cell types because pure populations of kidney-derived primary cell cul-

tures have not been available. In this regard, an established kidney epithelial cell line which stably propagates its differentiated characteristics would be useful. The availability of such a cell line would allow the unequivocal assignment of function to cell type and permit genetic as well as biochemical manipulation.

The cell line Madin-Darby canine kidney (MDCK) was derived from the kidney of a normal cocker spaniel without intentional exposure to carcinogens (9). When cultured on a glass surface, the cells have been shown to form an epithelial monolayer interspersed with multicellular hemispherical blisters (16, 17). The walls of the blisters and the contiguous monolayers on glass consisted of polarized epithelia with microvilli facing the medium-bathed surface. Time-lapse photography and studies with inhibitors of the Na^+ , K^+ -ATPase led to the suggestion that blister formation was due to active fluid transport which occurred vectorially from the mucosal (top) to the serosal (bottom) surface of the epithelium (17). Recent studies have confirmed and extended this conclusion (4, 22). It was shown that an MDCK cell monolayer cultured on a freely permeable support and placed in a Ussing chamber generated a transepithelial electrical potential. Net water flux, apical to basolateral, was also demonstrated.

Hemicyst formation in the MDCK cells is apparently regulated by cyclic AMP (36). Dibutyryl cAMP and/or papaverine increased the size and number of hemicysts in monolayer cultures over a 24-h period. The prospect of a physiologically relevant regulatory response provided part of the motivation for the hormonal studies presented here. In this regard, the MDCK cells were examined for their reaction to various hormones which regulate kidney function. By the criterion of adenylate cyclase stimulation, the cell line was shown to respond to vasopressin, oxytocin, prostaglandins, glucagon, and cholera toxin. The physiological implications of these observations are discussed.

Like many other tissue culture cell lines, MDCK cells have been reported to have acquired certain abnormal cytological characteristics, including abnormal mitotic figures and large nuclei (17). In addition, they had the ability to form small metastatic lesions when injected into chick embryos. Previous publications from this laboratory have served to further characterize the *in vivo* and *in vitro* growth properties of the MDCK line (32, 33). These cells do not proliferate when

injected subcutaneously into adult athymic nude mice, but they can be recovered in a viable state up to 2 mo post injection. By contrast, when MDCK cells are injected into baby nude mice, continuous nodule growth is observed for ~6 wk up to the time when adulthood is attained. The present report includes an electron microscope analysis of the MDCK cell nodules. The results show that these cells retain the ability to generate fluid-filled glands within the nodules which have certain morphological traits of intact kidney tubules.

MATERIALS AND METHODS

Materials

Tissue culture dishes were obtained from Lux Scientific Corp., Newbury Park, Calif. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Grand Island Biological Co., Grand Island, N. Y. (GIBCO). ^{14}C -cyclic AMP, ^3H -cyclic AMP, and α - ^{32}P -ATP were obtained from New England Nuclear, Boston, Mass. Isobutylmethylxanthine (IBMX), 3',5'-cyclic AMP, dibutyryl 3',5'-cyclic AMP, ATP, GTP, phosphocreatine, and phosphokinase were all purchased from Sigma Chemical Co., St. Louis, Mo. Lysine vasopressin was obtained from both Sigma and Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. (the two preparations yielded identical results). Arginine vasopressin, deoxycorticosterone, aldosterone, and hydrocortisone were obtained from Sigma, while parathyroid hormone (bovine), calcitonin (salmon), angiotensin II, and glucagon (bovine) were Calbiochem products. Cholera toxin was a gift of Dr. Nathan Kaplan, and prostaglandins were the gift of Dr. John Pike of the Upjohn Co., Kalamazoo, Mich. Prolactin (National Institutes of Health) and purified EGF were the gift of Dr. Gordon Sato. Dowex resins were purchased from Bio-Rad Laboratories, Richmond, Calif.; alumina was obtained from Sigma. All other materials were of the highest available purity.

Cell Cultures

The MDCK cell line was obtained from two sources. The sub-line obtained from Dr. John Holland was originally from the American Type Culture Collection and failed to form hemicysts under the usual culture conditions. In other respects, it had morphological and biochemical characteristics identical to those of the MDCK sub-line obtained from the Naval Biomedical Research Laboratory, Berkeley, Calif. The two sub-lines had identical hormone responses, and the former was employed in the current work. Cell cultures were maintained subconfluently after a 50 \times dilution of near confluent cultures in DMEM containing 200 U/liter penicillin, 0.2 mg/liter streptomycin (Calbiochem), and 10%

fetal bovine serum (GIBCO) in a 5% CO₂ incubator at 37°C. For determination of cyclic AMP production, cells were plated at 5 × 10³ cells per cm² in 35-, 60-, or 150-mm dishes and allowed to grow to confluence (8 d) with changes of medium on days four and six. When dibutyryl cyclic AMP was to be added to the plates, the cells were allowed to grow for 14 d with an additional medium change at day 10 before use. Although both MDCK lines were free of mycoplasma contamination when examined by autoradiography, mycoplasma was detectable in both when scanning electron microscopy or uridine/uracil incorporation tests were employed (30).

The MDBK (bovine) cell line was obtained from the American Type Culture Collection. It was also maintained subconfluently in DMEM with 10% fetal bovine serum. For analysis of cyclic AMP production, this cell type was plated under the conditions described above for MDCK cells and used at confluence. This cell line was free of mycoplasma by the uridine/uracil test.

Determination of Extracellular

Cyclic AMP Production

35-mm dishes of confluent MDCK monolayers were washed twice with 2 ml of phosphate-buffered saline (PBS) and once with 2 ml of DMEM without serum before addition of 1 ml of prewarmed DMEM containing the test agents. Dishes were incubated in a 37°C incubator with 5% CO₂ in air for the designated times before the extracellular fluid was collected, boiled for three minutes, and frozen until cyclic AMP analysis could be performed. Representative plates were analyzed for protein by the method of Lowry et al. (19) after removal of cells with a rubber policeman and homogenization. Confluent monolayers on 35-mm dishes contained ~1 mg of protein.

Cyclic AMP levels were measured by the filter binding assay of Gilman (11). Binding protein was prepared from fresh beef muscle through the DEAE-column step. Active fractions were pooled and stored in 2-ml aliquots at -70°C until used. Filters were dried and counted in 5 ml of a toluene scintillation mixture in a Beckman scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). cAMP determinations were routinely made in duplicate and averaged. Reproducibility of individual assays was normally within 10%. Confirmatory radioimmunoassays for cyclic AMP (Collaborative Research Inc., Waltham, Mass.) were performed on selected samples (31).

Determination of Intracellular

Cyclic AMP

Analysis of intracellular levels of cyclic AMP required purification due to low concentrations present and to the interference of intracellular protein with the Gilman assay. 60-mm dishes of confluent MDCK monolayers were washed twice with 5 ml of PBS and once with

DMEM. Then, prewarmed, pre-equilibrated DMEM (2.5 ml) containing the test agents was added to the plates, and cells were incubated as described above. At the appropriate times, extracellular fluid was collected as a control and the plates were washed three times with 5 ml of PBS at room temperature in <10 s before the addition of 2.5 ml of ice-cold 1% perchloric acid containing 3,000 cpm ¹⁴C-cyclic AMP to monitor recoveries. Purification of the nucleotide was accomplished by the anion exchange method described by Johnson (14). After 2.5 h of extraction, the fluid was placed in a test tube and neutralized with 1 N KOH for 15 min in ice before centrifugation in a Sorvall RC-3 (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) for 10 min at 2,500 g. The supernate was carefully removed and passed through columns of Dowex AG1-X8, 200-400 mesh (formate form) equilibrated with 0.1 N formic acid. Samples were eluted with 5 ml of 2 N formic acid, frozen, and lyophilized overnight. After resuspension in 0.5 ml of water, two samples of 200 μl were analyzed for their cyclic AMP content by the Gilman assay with 1 mM EDTA included. The remaining 100 μl were counted in a Beckman scintillation counter to determine their ¹⁴C-cyclic AMP content, using a Triton-toluene scintillant mixture. Recoveries were typically 55-65%. Representative plates were also analyzed for protein content as described above.

Adenylate Cyclase Assays

For measurement of adenylate cyclase activity *in vitro*, a crude membrane preparation was employed. Several 150-mm dishes were washed three times with 10 ml of PBS. The cells were subsequently scraped off the plates with a rubber policeman in 5 ml of a Tris-sucrose buffer consisting of 5 mM Tris-HCl, 0.25 M sucrose, 3 mM MgCl₂, and 1 mM EDTA, pH 8.0. Cells were then homogenized at 0°C with a 30-ml Thomas homogenizer (Arthur H. Thomas Co., Philadelphia, Penn.), using a drill press. After centrifuging at 40,000 g for 40 min, the pellet was resuspended in Tris-HCl buffer without sucrose, rehomogenized, and recentrifuged. Final resuspension was made in 1-2 ml of 5 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EDTA, pH 8.0 (except when the activity-pH profiles were determined). The crude preparation was then homogenized by hand in a 10-ml Teflon homogenizer before addition to the adenylate cyclase assay mix. Protein was measured by the method of Lowry et al. (19).

The basic adenylate cyclase assay mix consisted of 100 mM Tris-HCl, pH 8.0, 1.6 mM MgCl₂, 1 mM phosphocreatine, 10 μg/ml creatine phosphokinase, 0.1 mM GTP, 1 mM ATP, 50-150 micrograms of crude membrane protein, 0.1 mM IBMX, 0.2 mM EDTA and the test substance in a 0.1 ml volume. Samples were kept in ice until the assay was begun by placing the tubes in a 37°C water bath. The assay was terminated by boiling the tubes for 3 min. The absence of ATP, MgCl₂, or IBMX resulted in a total loss of detectable cyclic

AMP. Two methods of analysis were used for determining adenylate cyclase activity. The Gilman assay was employed after neutralization of the sample as described above, using 20 μ l samples. A method employing α - 32 P-ATP (39) proved more reliable when the activity was low. However, similar results were obtained by the two techniques when either prostaglandin E₁ or vasopressin was used to stimulate the enzyme. For the second procedure, α - 32 P-ATP ($\sim 10^9$ cpm) was included in the assay mix. After boiling the samples, 0.5 ml of a buffer containing 2.5 mM cyclic AMP, 8 mM ATP, and 5×10^4 cpm of 3 H-cAMP to monitor recoveries was added to each tube. Then 0.2 ml of H₂O and 0.2 ml of 1.5 M imidazole buffer, pH 7.5, were added before the sample was applied to a Dowex 50W-X8 column. The cyclic nucleotide was eluted with 5 ml of water. The first 2-ml aliquot was discarded, and the final 3-ml aliquot was collected and applied to an alumina column (3 ml bed vol) (29). The run through and 1 ml of the 0.1 M imidazole buffer wash was caught in scintillation vials, and the samples were counted in a Triton-toluene scintillation mixture.

Enzyme Assays

Most assays for the kidney specific enzymes studied have been described. These include the in vitro assay of the ouabain-sensitive Na⁺, K⁺-ATPase, measured in isolated membrane preparations from disrupted cells (15) ecto-p-nitro-phenylphosphate phosphatase (8), and ecto-leucine aminopeptidase (18). P-nitrophenyl phosphate (8 mM) in a saline buffer containing 50 mM glycine pH 9.0 at 37°C was used for the phosphatase assay by adding 1-ml aliquots of the solution to monolayers of MDCK on 35-mm dishes. The aminopeptidase measurements on whole cells were conducted in PBS, using 5 mM leucine p-nitroaniline. The aminopeptidase was also detected histochemically with fluorescein-labeled antileucine aminopeptidase antibody (18), generously supplied by Dr. Daniel Louvard. The degradation of trehalose and maltose (28) was attempted with a crude membrane preparation as described above, using a glucostat reagent set (Worthington Biochemical Corp., Freehold, N. J.) for detection of liberated glucose.

Electron Microscope Analyses

MDCK nodules from nude mice were dissected into 1 mm³ blocks and fixed in glutaraldehyde (2% in 0.1 M potassium phosphate buffer, pH 7.4) for 2 h at 4°C. They were rinsed with fresh cold buffer for a period of 2 h and then postfixed for 2 h with 2% OsO₄ in 0.1 M phosphate buffer. Postfixation and successive dehydration in graded concentrations of ethanol was performed at 4°C. The solution was allowed to reach room temperature during the 70% ethanol step. After the appropriate steps in propylene oxide and Epon 812, the tissue was polymerized at 60°C under vacuum for a period of 24–48 h. Staining with 2% uranyl acetate and Reynolds'

lead citrate followed sectioning on a Reichert microtome (American Optical, Buffalo, N. Y.). The sections were examined on a Philips EM-300 (12).

RESULTS

Enzyme Activities Associated with MDCK Cells Grown In Vitro

When MDCK cells were grown in tissue culture, they formed a continuous epithelial sheet covering the surface of the plate. Electron microscope examination of these cells revealed structural polarity with microvilli facing upward toward the culture medium and smooth membranes apposed to the solid support (unpublished observation). Individual cells were joined by continuous tight junctions, confirming the observations of Misfeldt et al. (22).

Several differentiated biochemical characteristics of these cells were examined. The cells exhibited high ecto p-nitrophenylphosphate phosphatase (2.5 nmol p-nitrophenol formed/min/mg, protein at 37°C) and leucine aminopeptidase activities (16 nmol p-nitroaniline/min/mg protein at 37°C). Cell-free extracts derived from the MDCK cells possessed high Na⁺, K⁺-dependent ATPase activity which was sensitive to inhibition by ouabain (1). Attempts to demonstrate the presence of several proximal renal tubular-specific enzymatic markers (maltase, trehalase [each <0.5 μ mol/h/mg protein at 37°C] Na⁺-dependent glucose uptake, p-amino hippurate uptake) were negative. 22 Na⁺ flux measurements with intact cells revealed an amiloride-sensitive Na⁺ channel, although fairly high concentrations of this inhibitor ($>10^{-5}$ M) were required to block Na⁺ entry when the extracellular Na⁺ concentration was 1 mM (5, 10, 34, 35). The mechanism of Na⁺ transport in the MDCK cells will be the subject of separate reports.

Response of Adenylate Cyclase in MDCK Cells to Hormones

When vasopressin, prostaglandin E₁ or E₂ or glucagon in the presence of IBMX was added to confluent MDCK monolayer cultures, increased production of cyclic AMP in the extracellular medium was observed (Fig. 1). Prostaglandin E₁ stimulated the production of cyclic AMP approximately five times more effectively than vasopressin and ten times more effectively than glucagon.

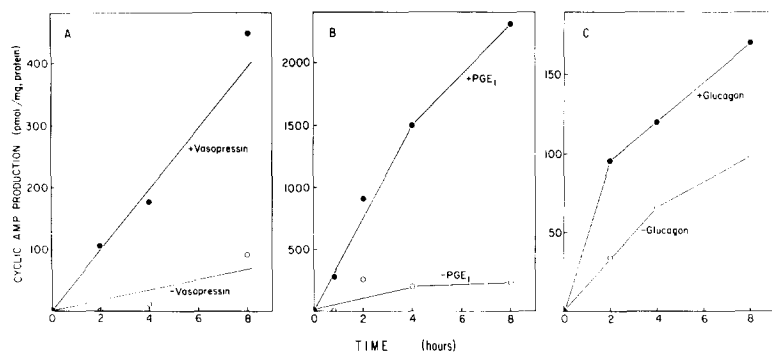


FIGURE 1 Production of extracellular cyclic AMP in response to various hormonal agents as a function of time. Confluent monolayers in 35-mm dishes were incubated in DMEM at 37°C in the presence of 0.1 mM IBMX. Hormone concentrations were: vasopressin, 1 μ M, PGE₁, 10 μ M, and glucagon, 70 μ M. The rate of a cAMP production was nearly linear for 8 h as measured by the binding assay of Gilman.

The rise in the intracellular concentration of cyclic AMP as a function of time was markedly divergent from the pattern observed for production of the nucleotide in the medium. As shown in Fig. 2, the cyclic AMP concentration increased very rapidly, reaching a peak value in 5 min in the case of both vasopressin and prostaglandin. Spikes of intracellular cyclic nucleotides are characteristic of many hormone responses, as is the activation of certain transport systems by intracellular cyclic AMP (6, 27).

Fig. 3 shows dose-response curves for a number of pharmacological and hormonal agents. IBMX (Fig. 3A) caused an augmentation in the basal level of the cyclic nucleotide and a corresponding effect in the presence of vasopressin. Although the total amount of the cyclic AMP increased as the IBMX concentration was raised, the relative stimulation by the hormone over the background level remained constant. The hormone receptor exhibited a marked lack of specificity, yielding similar dose-response curves for the lysine ($K_m = 3 \times 10^{-8}$ M) and arginine ($K_m = 1 \times 10^{-8}$ M) forms as well as for oxytocin (Fig. 3B and C). Dose-response curves for vasopressin stimulation of adenylate cyclase in other kidney systems have been found to parallel those reported here (7).

Prostaglandins E₁ and E₂ increased cyclic AMP levels in a sharply defined manner (Fig. 3D). Table I documents the specificity of the prostaglandin response. The E prostaglandins were more effective than prostaglandin A₁ or A₂ and were much more effective than prostaglandin B₁ or F_{2 α} .

Fig. 3E defines the dose-response curve for

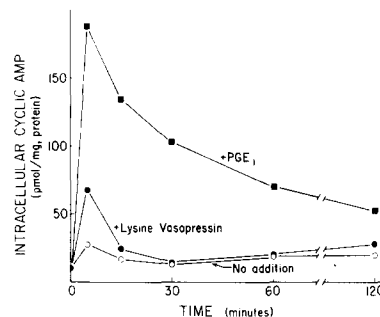


FIGURE 2 Production of intracellular cyclic AMP in response to Prostaglandin E₁ and vasopressin as a function of time. 60-mm dishes were incubated as in Fig. 1. Cells were treated with perchloric acid which was removed by neutralization before passage over a Dowex AG1-X8 anion exchange column as described under Materials and Methods.

glucagon. The small increase was first detectable at $\sim 10^{-5}$ M. Cholera toxin was a very potent effector of the adenylate cyclase system in the MDCK cells (Fig. 3F). Further incubation led to an even greater response due to the time-dependent cholera toxin activation process. It is therefore clear that none of the hormonal agents—i.e., prostaglandin, glucagon, or vasopressin—were capable of fully activating adenylate cyclase in the MDCK cells.

In Vitro Adenylate Cyclase Experiments

To further characterize the hormone responses, a crude membrane pellet was prepared from monolayer cultures of MDCK cells. Cyclic AMP production was measured either by the Gilman assay or by the α -³²P-ATP technique

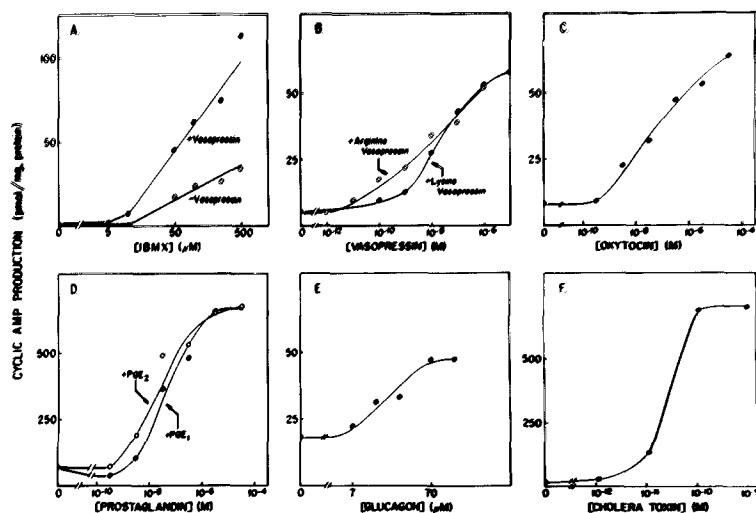


FIGURE 3 Production of extracellular cyclic AMP as a function of hormone concentration. Monolayers were treated as in Fig. 1 and assayed after an incubation period of 2 h, except for the glucagon samples which were allowed to incubate for 4 h. The IBMX concentration was 0.1 mM in Fig. 4 B-F. Cyclic AMP production is reported in pmol/h/mg, protein. Differences in the scales of the individual graphs should be noted.

TABLE I
Cyclic AMP Production by MDCK Cells in Response to Various Prostaglandins

Agent	Cyclic AMP Production (pmol/h/mg, protein)		
	3×10^{-3} M	3×10^{-6} M	3×10^{-7} M
Prostaglandin A ₁	361	170	49
A ₂	392	333	75
B ₂	ND	108	17
E ₁	544	484	356
E ₂	513	481	454
F _{2α}	173	66	39

Cyclic AMP production in the absence of Prostaglandin amounted to 33 pmoles/h/mg, protein and was subtracted from all values recorded above.

outlined under Materials and Methods. The two methods gave nearly identical results. Time courses of cyclic AMP production are shown in Fig. 4, and, on the basis of these data, 15–30 min was chosen as an appropriate time for assaying cyclic AMP. Fig. 5 demonstrates the $MgCl_2$ dependency of the adenylate cyclase activity at 1 mM ATP. A sharp activity peak with an optimum at 1.6 mM $MgCl_2$ was noted for both the basal and prostaglandin-stimulated assays. A broad activity-pH profile was observed with a pH optimum at 8.0 (data not shown). Similar results have been reported for the pig kidney

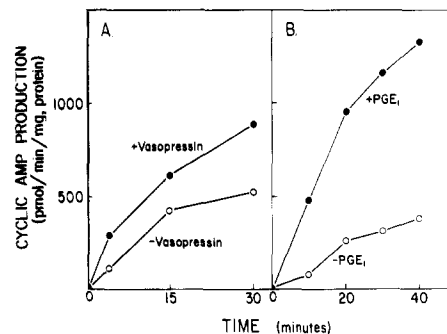


FIGURE 4 Time course of the adenylate cyclase response in vitro. Cells were scraped off of the dishes and a membrane fraction was collected by centrifugation. Assay was conducted at 37°C in the presence of 1 mM ATP, 0.1 mM GTP, 0.1 mM IBMX, 1.6 mM $MgCl_2$, 100 mM Tris-HCl buffer, pH 8, and an ATP regenerating system. Samples were boiled 3 min and assayed by the Gilman method (11). Hormone concentrations used: vasopressin, 1 μ M, PGE₁, 10 μ M. GTP, at 0.1 mM, effected a twofold increase in activity. ~80 μ g of protein was included in each assay tube.

enzyme (3). In addition, a marked effect of GTP on the activity of adenylate cyclase was noted. The twofold increase in both basal and hormone-stimulated activity was maximal with 0.1 mM GTP (data not shown). Finally, dose-response curves of vasopressin and PGE₁ showed that stimulation of adenylate cyclase was not detectable

ble until 10^{-7} M hormone was present. The relative increase in cyclic AMP production by prostaglandin E (threefold) was substantially higher than that by vasopressin (50%).

Responses to Other Hormones

A variety of additional hormones with a kidney-related site of action were tested for their effects on cyclic AMP Levels. As shown in Table II, parathyroid hormone, calcitonin, angiotensin, and prolactin were not effective agents. Isoproterenol was occasionally able to raise cyclic AMP production slightly, but the effect was not reproducible. By way of comparison, cyclic AMP

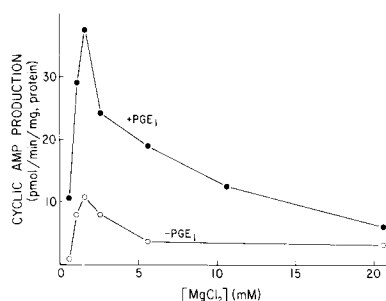


FIGURE 5 Dependency of adenylate cyclase on $MgCl_2$. Cyclic AMP production was determined by the Gilman assay after a 15-min incubation period at $37^\circ C$. The ATP concentration was 1 mM. The assay conditions were otherwise similar to those reported in Fig. 4 and under Materials and Methods.

production for another kidney epithelial cell line, MDBK (20), was examined for its hormonal specificities. As summarized in Table II, this cell line has a distinct pattern of hormone responsiveness. For example, MDBK did not respond appreciably to vasopressin but showed marked responses to catecholamines and prostaglandins.

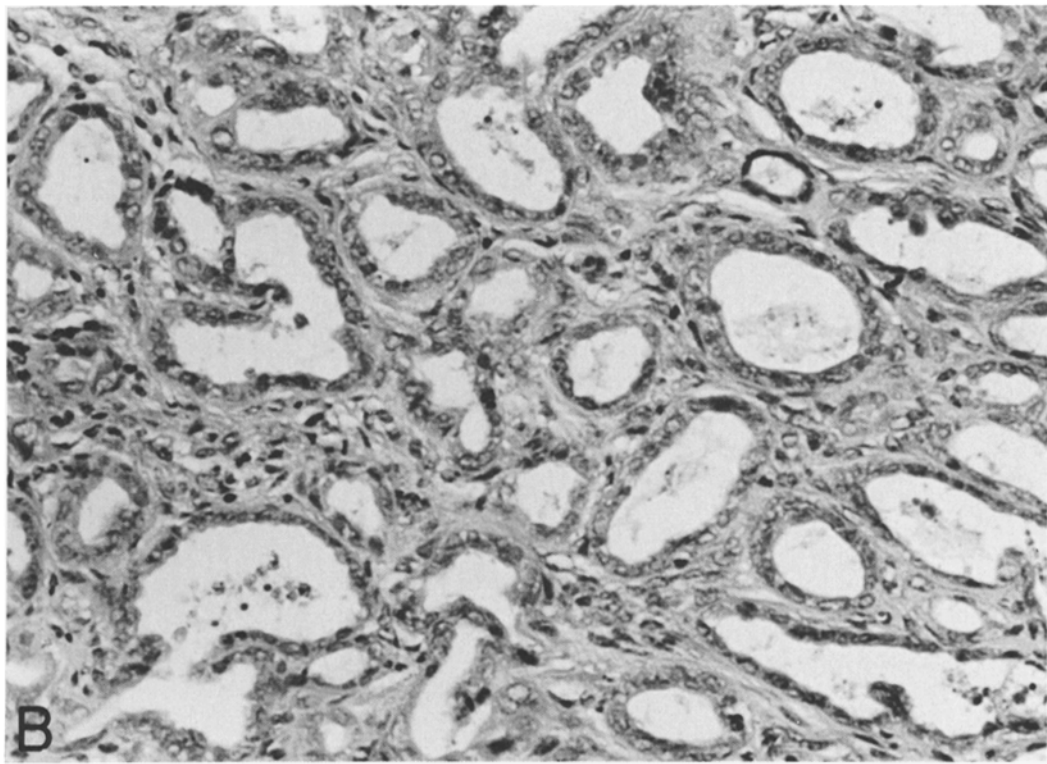
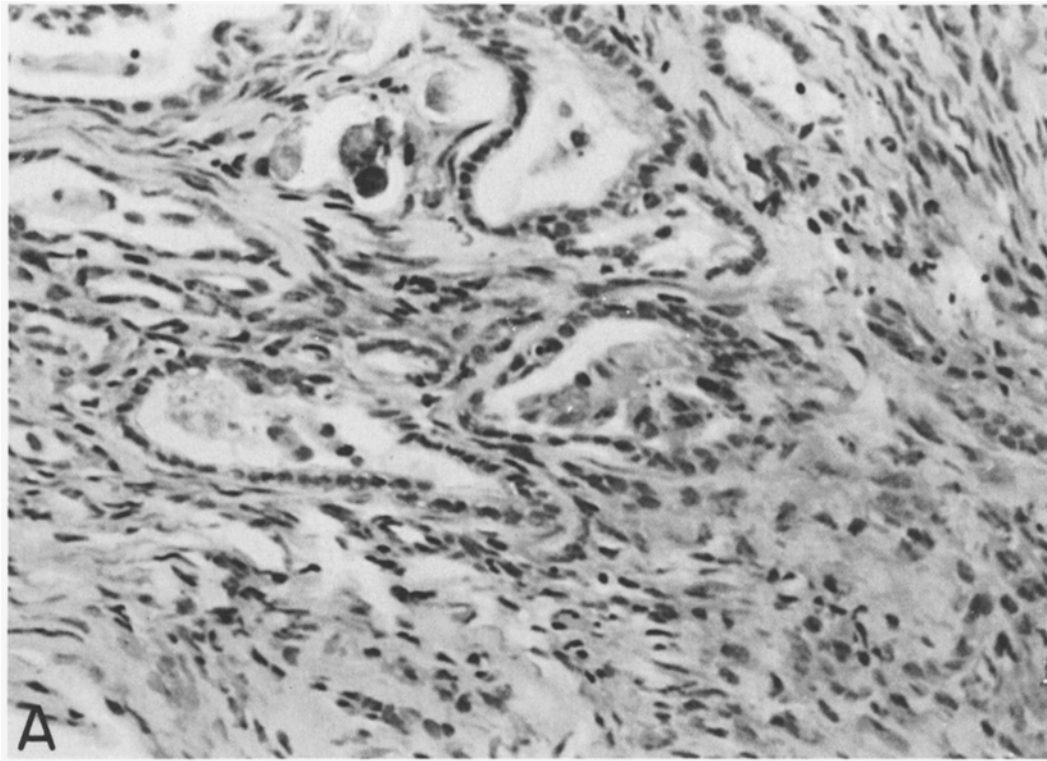
Histological and Electron Microscope Characterization of MDCK Nodules from Nude Mice

As reported previously (32), MDCK cells form nodules when injected subcutaneously into baby athymic nude mice but not when injected into adult animals. Fig. 6 shows the histology of nodules removed (A) from a growing (3-wk-old) host, and (B) from an adult (4-mo-old) host. In both cases, 10^6 MDCK cells were injected subcutaneously 1 d after birth. As shown in Fig. 6, nodules recovered from the growing host contained mostly glandular epithelium in a dense stroma. While some of the glands were large, well-formed, and lined by a monolayer of columnar epithelium (presumably the injected MDCK), others were of a somewhat distorted or incomplete configuration with smaller lumens. Contiguous with these collections of epithelial cells, and especially prominent in the immature nodule (Fig. 6A), were short, ill-defined cords of cells as well as single cells. Histological analy-

TABLE II
Cyclic AMP Production by MDCK and MDBK Cells in Response to Various Hormonal Agents

Agent	Concentration	Cyclic AMP production (pmol/h/mg, protein)	
		MDCK	MDBK
None	—	2	17
IBMX	10^{-4} M	18	19
IBMX + Prostaglandin E_1	10^{-6} M	325	240
IBMX + Arg-vasopressin	10^{-6} M	76	30
IBMX + Isoproterenol	10^{-5} M	28	341
IBMX + Glucagon	7×10^{-5} M	55	ND
IBMX + Parathyroid hormone	10^{-8} M	26	25
IBMX + Calcitonin	10^{-8} M	20	23
IBMX + ACTH	0.5 U/ml	20	ND
IBMX + Prolactin	10^{-7} M	18	18
IBMX + Adenosine	10^{-4} M	12	ND
IBMX + Angiotensin II	10^{-5} M	17	ND
IBMX + Epidermal growth factor	10^{-6} g/ml	28	ND
Prostaglandin E_1	10^{-6} M	10	207
Arg-vasopressin	10^{-6} M	0	27

Cells were treated for 2–4 h with the agents before analysis for extracellular cyclic AMP.



sis also revealed that the epithelium is abnormal in its architectural and cytological appearances. Many cells have particularly large nuclei and nucleoli, and the tissue invades around nerves. The nodules can be characterized as well differentiated adenocarcinoma consistent with a renal tubular site of origin (K. Benirschke and J. Leighton, personal communication).

The fine structure of the nodules was revealed by electron microscopy. Fig. 7 shows an electron micrograph of a portion of a young (3-wk-old) nodule in the region of a fluid-filled gland. Lining it were MDCK cells, identifiable by their compact shape and by the presence of numerous microvilli (*MV*). In the immature nodule, microvilli lined the sac, but they were also found in apposition to other cells. The epithelial layer was clearly disorganized, with numerous MDCK cells completely surrounded by cellular and fibrous (*CF*) elements. Junctional complexes (*JC*) were poorly formed, and no distinct basement membrane could be seen.

An electron micrograph of a representative tubule within a mature MDCK nodule is reproduced in Fig. 8A. Several features are worthy of note. First, the fluid-filled gland is surrounded by a single layer of MDCK cells consisting of seven adjacent cells. All of the microvilli (*MV*) face the lumen (*L*). Second, the epithelial cell layer is completely surrounded on the basolateral surface by a distinct basement membrane (*BM*). The basolateral surface does not display microvillar structures. Third, a junctional complex (*JC*) is observed joining the adjacent epithelial cells which comprise the gland. This complex is located near the mucosal surface of the cells and does not usually continue along the entire boundary of the cell-cell contact region. A view of the junctional complexes at high magnification (Fig. 8B) revealed structures resembling both tight junctions (*TJ*) and adhering junctions (*AJ*). Mitochondria (*M*), Golgi elements (*G*) and multivesicular bodies (*MVB*) can also be seen in this electron micrograph. Finally, surrounding the tubule can be seen elongated fibroblasts (*F*) and large bundles of collagen fibers (*CF*), presumably derived from the host fibroblasts.

DISCUSSION

The MDCK cell line provides a useful model system for the study of salt and water reabsorption and its regulation in the kidney. The data presented herein confirm that the MDCK cells have retained several hormone receptors characteristic of kidney tissue. Vasopressin, prostaglandin E₁ and E₂, and, to some extent, glucagon, were capable of increasing cyclic AMP production in monolayer cultures containing a single cell type. The hormonal stimulation of cyclic AMP production was confirmed by in vitro analysis of adenylate cyclase activity.

It is well established that the mechanism of action of the antidiuretic hormones involves the production of cyclic AMP. Early studies (24) demonstrated that vasopressin increased the cyclic AMP levels of kidney slices, and that cyclic AMP and/or theophylline mimicked the effects of ADH. The molecular events leading to the increase in water permeability of the luminal membrane surface remain largely obscure, although it is thought to occur in the kidney by the classical activation of protein kinases or protein phosphatases by cyclic AMP. More recent studies have described the properties of the vasopressin-sensitive adenylate cyclases of the pig, rat, and bovine kidney. A half-maximal stimulation of the rat adenylate cyclase at $\sim 10^{-8}$ M vasopressin (26) is comparable to the results obtained in the whole-cell studies presented here, to that reported for the pig system (5×10^{-9} M) (3), and to that reported for beef kidney extracts (10^{-9} M) (2). Investigations with the beef enzyme also do not show sharp distinction between lysine and arginine vasopressin (2). Other properties of the cyclase appear to be quite similar to those reported in previous studies. Prostaglandin stimulation of adenylate cyclase was much more pronounced than that observed with vasopressin. Several investigators have reported effects of the prostaglandins in the kidney (13). Although the physiological functions and precise sites of action of prostaglandins in the kidney remain to be determined, effects of the hormone on salt and water transport in isolated collecting tubules

FIGURE 6 Histological cross-section through MDCK nodules formed in nude mice given neonatal injections. The nodules were excised and fixed with 10% formalin. H & E, $\times 100$. (A) The nodule was excised from a 3-wk-old nude mouse; (B) the nodule was excised from an adult nude mouse.

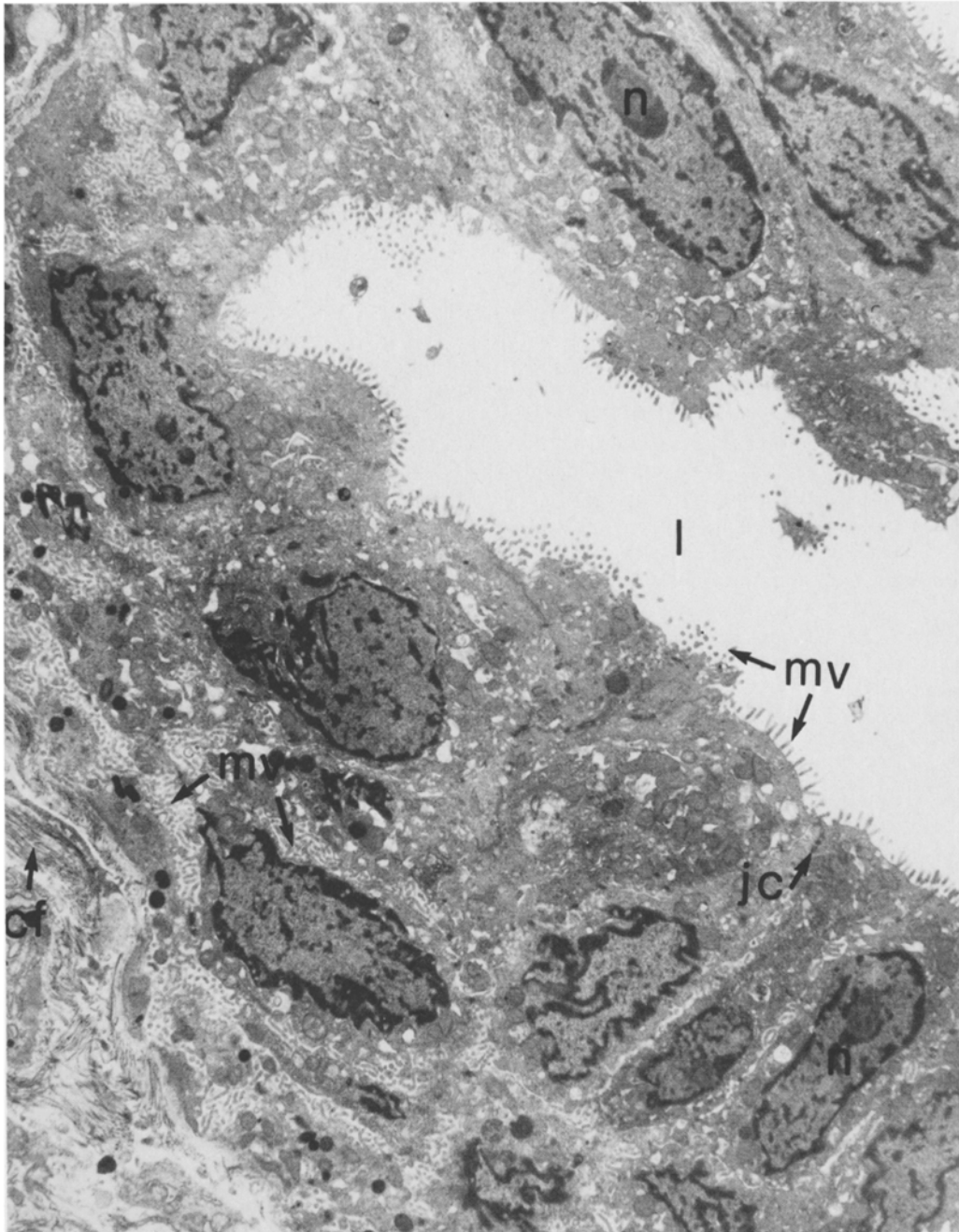


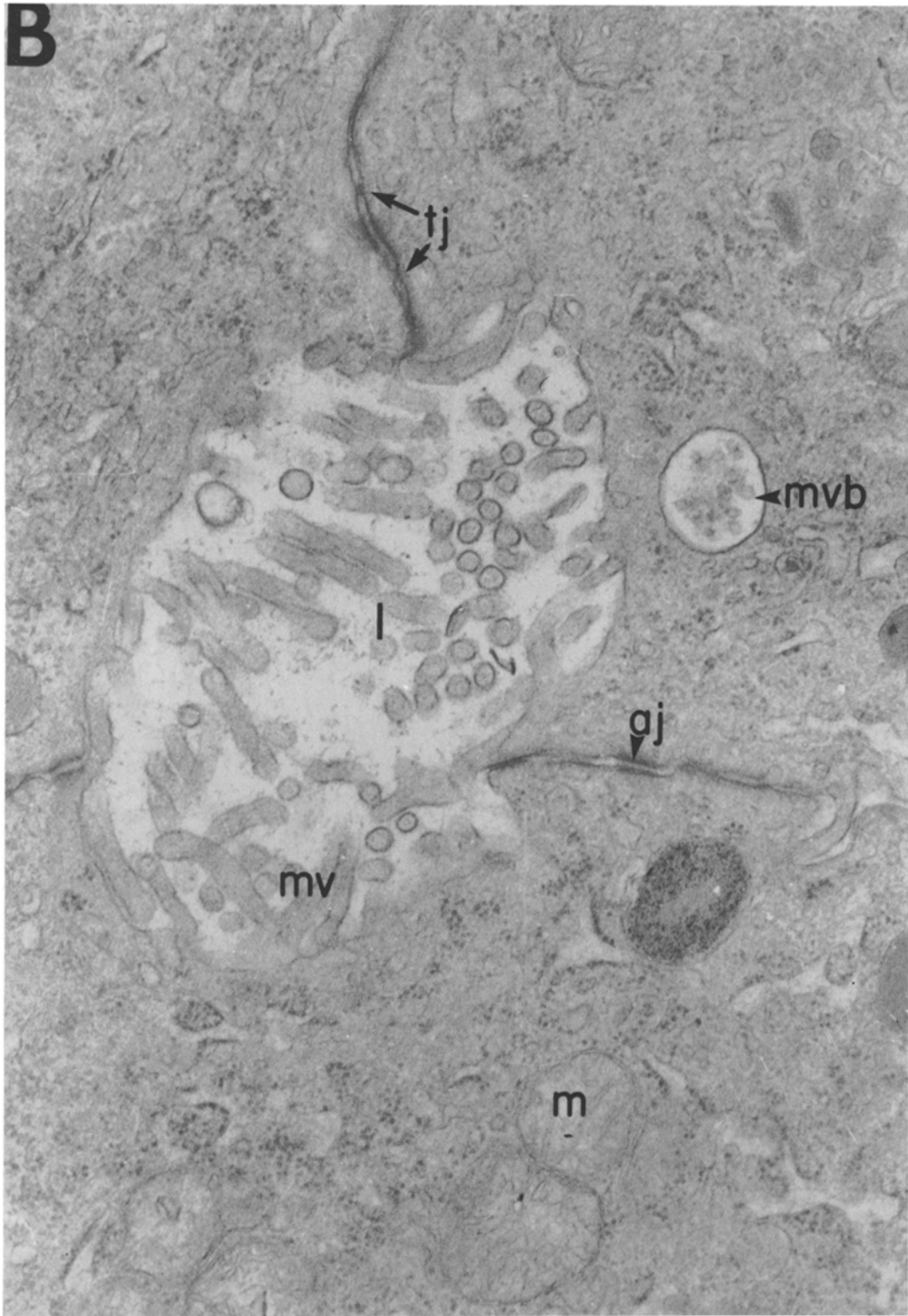
FIGURE 7 Electron micrograph of an internal portion of an immature MDCK nodule recovered from a growing nude mouse. Abbreviations are as follows: *L*, lumen; *MV*, microvilli; *N*, nucleus; *CF*, collagen fibers; *JC*, junctional complexes. $\times 5,300$.

have been documented and have led to the suggestion that they serve as modulators of vasopressin-induced changes in the water permeability of certain transporting epithelia (13). The

stimulatory effect of glucagon on cyclic AMP production in the MDCK cells was small. Glucagon has been reported to stimulate kidney adenylate cyclase (21, 23) and to increase the excre-



FIGURE 8 Electron micrographs of internal portions of a mature MDCK nodule recovered from an adult nude mouse. Abbreviations are as follows: *L*, lumen; *MV*, microvilli; *CF*, collagen fibers; *JC*, junctional complexes; *BM*, basement membrane; *F*, fibroblast; *N*, nucleus; *TJ*, tight junction; *AJ*, adhering junction; *MVB*, multivesicular body; *M*, mitochondria. (A) $\times 5300$; (B) $\times 23,000$.



tion of sodium and other electrolytes in the intact tissue (25).

The studies reported here suggest that MDCK cells not only possess differentiated kidney specific transport properties but also retain the ability to regenerate kidney tubular-like structures when present in an appropriate environment. Thus, when MDCK cells were injected into baby nude mice, nodules developed which contained two cell types: normal mouse fibroblasts which comprised ~90% of the solid nodule mass, and MDCK cells which formed epithelial sheets lining internal fluid-filled glandular structures that suggested an almost tubular arrangement. Electron microscope analysis showed that (a) all microvilli (on the mucosal surface) faced the lumen of the glands, (b) adjacent MDCK cells were joined by tight junctions, and (c) the serosal surfaces of the epithelial sheets were characterized by smooth membranes which were lined by a basement membrane (Fig. 8). In vitro cell aggregation studies showed that these cells exhibited adhesive properties which allowed them to form pure and mixed cell aggregates (unpublished results). These results lead to the suggestion that the MDCK cells retain (a) the capacity for both homotypic and heterotypic adhesion, (b) regional differentiation of the cell surface membranes, (c) sensitivity to host generated growth regulatory signals, and (d) potential for the regeneration of kidney tubule-like structures. Because these cells have escaped the restraints of mortality, they offer the potential for genetic as well as biochemical and physiological manipulation (35). They appear to provide excellent material for studies into the molecular details of kidney specific function and biogenesis.

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REFERENCES

1. ABAZA, N. A., J. LEIGHTON, and S. G. SCHULTZ. 1974. Effects of ouabain on the function and structure of a cell line (MDCK) derived from canine kidney. I. Light microscopic observations on monolayer growth. *In Vitro (Rockville)*. **10**:172-183.
2. BIRNBAUMER, L., and P. C. YANG. 1974. Studies on receptor-mediated activation of adenylyl cyclase. I. Preparation and description of general properties of an adenylyl cyclase system in beef renal medullary membranes sensitive to neurohypophysial hormones. *J. Biol. Chem.* **249**:7848-7856.
3. BOCKAERT, J., C. ROY, R. RAJERISON, and S. JARD. 1973. Specific binding of [³H]lysine-vasopressin to pig kidney plasma membranes. Relationship of receptor occupancy to adenylyl cyclase activation. *J. Biol. Chem.* **248**:5922-5931.
4. CERELIJO, M., E. S. ROBBINS, W. J. DOLAN, C. A. ROTUNNO, and D. D. SABATINI. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **77**:853-880.
5. CUTHBERT, A. W., and W. K. SHUM. 1976. Characteristics of the entry process for sodium in transporting epithelia as revealed with amiloride. *J. Physiol.* **255**:587-604.
6. DOORE, B. J., M. M. BASHOR, N. SPITZER, R. C. MAWE, and M. H. SAIER, JR. 1975. Regulation of adenosine 3':5'-monophosphate efflux from rat glioma cells in culture. *J. Biol. Chem.* **250**:4371-4372.
7. DOUSA, T. P., R. WALTER, I. L. SCHWARTZ, H. SANDS, and O. HECHTER. 1972. *Adv. Cyclic Nucleotide Res.* **1**:121-136.
8. EICHHOLZ, A. 1967. Structural and functional organization of the brush border of the intestinal epithelial cells. III. Enzymic activities and chemical composition of various fractions of tris-disrupted brush borders. *Biochim. Biophys. Acta.* **135**:475-482.
9. GAUSH, C. R., W. L. HARD, and T. F. SMITH. 1966. Characterization of an established line of canine kidney cells (MDCK). *Proc. Soc. Exp. Biol. Med.* **122**:931-935.
10. GIEBISCH, G. 1976. Renal physiology. *Ann. Rev. Physiol.* **38**:7-45.
11. GILMAN, A. G. 1970. A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**:305-312.
12. GORDON, G. B., L. R. MILLER, and K. G. BENSCH. 1963. Fixation of tissue culture cells for ultrastructural cytochemistry. *Exp. Cell Res.* **31**:440-443.
13. GRANTHAM, J. J., and J. ORLOFF. 1968. Effect of prostaglandin E₁ on the permeability response of isolated collecting tubules to vasopressin, adenosine 3',5'-monophosphate, and theophylline. *J. Clin. Invest.* **47**:1154-1161.
14. JOHNSON, R. A. 1972. Assay of cyclic AMP. In *Methods in Cyclic Nucleotide Research*. M. Chasin, editor. Marcel Dekker, New York. 1-27.
15. KYTE, J. 1971. Purification of the sodium- and potassium-dependent adenosine triphosphatase from canine renal medulla. *J. Biol. Chem.* **246**:4157-4165.
16. LEIGHTON, J., Z. BRADA, L. W. ESTES, and G. JUSTH. 1969. Secretory activity and oncogenicity of a cell line (MDCK) derived from canine kidney. *Science (Wash. D. C.)* **163**:472-473.
17. LEIGHTON, J., L. W. ESTES, S. MANSUKHANI, and Z. BRADA. 1970. A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. *Cancer* **26**:1023-1028.
18. LOUVAR, D., S. MAROUX, and P. DESNUELLE. 1975. Topological studies on the hydrolases bound to the intestinal brush border membrane II. Interactions of free and bound aminopeptidase with a specific antibody. *Biochim. Biophys. Acta.* **389**:389-400.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**:265-275.
20. MADIN, S. H., and N. B. DARBY, JR. 1958. Established kidney cell line of normal adult bovine and ovine origin. *Proc. Soc. Exp. Biol. Med.* **98**:574-576.
21. MELSON, G. L., L. R. CHASE, and G. D. AURBACH. 1970. Parathyroid hormone-sensitive adenylyl cyclase in isolated renal tubules. *Endocrinology*. **86**:511-518.
22. MISFELDT, D. S., S. T. HAMAMOTO, and D. R. PTELKA. 1976. Transepithelial transport in cell culture. *Proc. Natl. Acad. Sci. U. S. A.* **73**:1212-1216.
23. MULVEHILL, J. B., Y. S. HUI, L. D. BARNES, P. J. PALUMBO, and T. P. DOUSA. 1976. Glucagon-sensitive adenylyl cyclase in human renal medulla. *J. Clin. Endocrinol. Metab.* **42**:380-384.
24. ORLOFF, J., and J. S. HANDLER. 1964. The cellular mode of action of antidiuretic hormone. *Am. J. Med.* **36**:686-697.
25. PULLMAN, T. N., A. R. LAVENDER, and L. AHO. 1967. Direct effects of glucagon on renal hemodynamics and excretion of inorganic ions. *Metab. Clin. Exp.* **16**:358-373.

26. RAJERISON, R., J. MARCHETTI, C. ROY, J. BOCKAERT, and S. JARD. 1974. The vasopressin-sensitive adenylate cyclase of the rat kidney. Effect of adrenalectomy and corticosteroids on hormonal receptor-enzyme coupling. *J. Biol. Chem.* **249**:6390-6400.
27. RINDLER, M. J., M. M. BASHOR, N. SPITZER, and M. H. SAIER, JR. 1978. Regulation of adenosine 3'5'-monophosphate efflux from animal cells. *J. Biol. Chem.* **253**:5431-5436.
28. SACKTOR, B. 1968. Trehalase and the transport of glucose in the mammalian kidney and intestine. *Proc. Natl. Acad. Sci. U. S. A.* **60**:1007-1014.
29. SALOMON, Y., C. LONDOS, and M. RODBELL. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541-548.
30. SCHNEIDER, E. L., E. J. STANBRIDGE, and C. J. EPSTEIN. 1974. Incorporation of ³H-uridine and ³H-uracil into RNA. A single technique for the detection of mycoplasma contamination in cultured cells. *Exp. Cell Res.* **84**:311-318.
31. STEINER, A. L., C. W. PARKER, and D. M. KIPNIS. 1972. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J. Biol. Chem.* **247**:1106-1113.
32. STILES, C. D., W. DESMOND, L. M. CHUMAN, G. SATO, and M. H. SAIER, JR. 1976. Growth control of heterologous tissue culture cells in the congenitally athymic nude mouse. *Cancer Res.* **36**:1353-1360.
33. STILES, C. D., W. DESMOND, L. M. CHUMAN, G. SATO, and M. H. SAIER, JR. 1976. Relationship of cell growth behavior *in vitro* to tumorigenicity in athymic nude mice. *Cancer Res.* **36**:3300-3305.
34. STOFF, J. S., F. H. EPSTEIN, R. NARINS, and A. S. RELMAN. 1976. Recent advances in renal tubular biochemistry. *Ann. Rev. Physiol.* **38**:46-68.
35. TAUB, M. 1978. Isolation of amiloride resistant clones from dog kidney epithelial cells. *Somatic Cell Genetics.* **4**:619-625.
36. VALENTICH, J. D., R. TCHAO, and J. LEIGHTON. 1976. Functional control of transporting epithelia by cAMP and divalent cations. *J. Cell Biol.* **70**(2, Pt. 2):330a. (Abstr.).