Electrophysiologic Study of Rabbit Proximal Tubular Cell Monolayers in Primary Culture

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Abstract. **Primary cultures of renal cortical cells prepared by selective sieves have been found to display some characteristics of renal proximal tubular epithelium but their site of origin has not been confirmed by electrophysiologic studies. Cells were cultured in a defined medium on collagen gels. Confluency was approached after 7–10 days but gels were found to have zero transepithelial resistance unless they were allowed to contract spontaneously. With the appearance of a nonzero resistance, there was a change in morphology to a more columnar cell with better developed microvilli. These structural features were particularly prominent in clusters of proliferating cells observed on and around remnants of original tubules embedded in the gel. In noncontracted cultures there was no focal cell clustering and cells were squamous-like with rudimentary microvilli, similar in appearance to cells grown on plastic culture dishes. Measurements made in contracted monolayers yielded an average transepithelial resistance of 6.5 Ω cm^2, a spontaneous transepithelial potential difference of +0.9 mV, measured with respect to the serosa, and an apical membrane potential of −75 mV when cells were bathed in 0.4 m*M* K and −49 mV when cells were bathed in 4 m*M* K media. Mucosal protamine (50 μg/ml) increased transepithelial resistance by 22%, suggesting that the epithelial cell tight junctions were responsive to external stimuli. Monolayers were anion selective, giving a dilution potential (lumen-directed NaCl gradient) of −2.6 mV with respect to the serosa.**

These experiments show that primary culture of rabbit renal cortical cells separated by differential sieves displays electrophysiologic and morphologic characteristics of a proximal renal tubular epithelium. Confluency and attainment of differentiated morphology and function are promoted when monolayer cells are not bound to an unyielding substrate. [P.S.E.B.M. 1989, Vol 190]

Cell culture of various renal epithelial cells has been the subject of intensive study (1). Misfeldt *et al.* (2) successfully measured transepithelial physiologic parameters *in vitro* in monolayers of an established dog kidney cell line (MDCK) grown on Millipore filter supports. Subsequently, the vigorous growth characteristics of established cell lines, where cells may be transformed, allowed polarized transport studies in several other kidney cell lines from mammalia and amphibia (3–5). More recently, even primary kidney cell culture monolayers, which are generally more fastidious and require individualized media and support matrices, have been studied (1, 6–8). Primary cell culture, in contrast to the culturing of transformed cell lines, has the advantage of yielding monolayers with properties more similar to those found *in vivo*, provided, of course, that cells are adequately differentiated. Monolayer culturing allows experiments to be done with Ussing-type chambers, which afford easier handling than renal micropuncture or isolated perfused tubule preparations, precise control of the bathing media, and accurate measurements of the transport of charged and uncharged chemical species.

Primary cultures of kidney proximal tubule cells were first grown as epithelial monolayers by Chung *et al.* (1). This preparation has been the subject of a number of studies which have demonstrated properties characteristic of proximal tubule cells *in vivo*. The

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cultures have been found to contain angiotensin-converting enzyme in the cell membranes (9) and high activities of the brush border enzymes, alkaline phosphatase, γ-glutamyl transpeptidase, and leucine aminopeptidase (1). They display phosphate transport (10) and glucose uptake (11) which are sodium dependent. In addition, parathyroid hormone stimulates cAMP synthesis while arginine vasopressin and calcitonin do not (1). Finally, prostaglandin and thromboxane synthesis have been demonstrated (12). However, electrophysiologic parameters probably best characterize the site of origin of an epithelium and in the case of a primary culture is essential not only to identify origin but also purity of the monolayer. Bello-Reuss and Weber (6) have described the properties of monolayers grown from rabbit proximal tubular cells isolated by a different method. Hence, the objective of this study was to determine whether primary cultures of rabbit proximal tubule cell monolayer harvested according to the method of Chung *et al.* (1) have electrophysiologic and morphologic properties consistent with that of proximal tubular epithelium.

Materials and Methods

Culture. Briefly, kidneys were excised from freshly sacrificed New Zealand White rabbits and placed in medium (Dulbecco's modified Eagle's (DME)/F-12) consisting of equal mixtures of DME medium and Ham's F-12 containing 25 m*M* Hepes, penicillin G (100 units/ml), and streptomycin (100 μg/ml). The renal artery was cannulated and kidneys perfused with phosphate-buffered saline until free of blood. This was followed with infusion of about 3 ml of 0.5% magnetic iron hydroxide in phosphate-buffered saline until the kidney was gray-black. After decapsulating the kidney, the cortex was cut into pieces and placed in a Dounce tissue grinder and homogenized with four strokes of a loose (B-type) pestle. Tissue fragments were then passed through two nylon sieves (253- and 83-μm pore size) in series. The tissue retained on the 83-μm sieve was suspended in DME/F-12 medium. Iron-containing glomeruli remaining in the suspension were removed with a magnetic stirring bar. Tubular fragments were then incubated with trypsin inhibitor at a final concentration of 0.1% (GIBCO Laboratories, Grand Island, NY) and collagenase Type IV at a final concentration of 1 mg/ml (Sigma Chemical, St. Louis, MO) for 2 min and centrifuged at 1000*g* for 3 min. The supernatant was discarded and the tissue was resuspended in medium. This procedure was repeated twice, after which the fragments were plated in 12-well culture dishes over a collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) disk with no embedded support, prepared according to the method of Lowy *et al.* (13). However, disks were cast with a cotton or silk thread embedded in the outer circumference. This served to retard the subsequent cell-mediated contraction of the gels and maintain them in a flatter, less wrinkled state. The medium was DME/F-12 containing insulin (5 μg/ml), transferrin (5 μg/ml), and hydrocortisone (5×10^{-8} *M*).

Cultures were incubated at 37°C in 5% CO_2. The medium was changed after 24 hr and every 4–5 days thereafter. Confluency was reached in 7–10 days. In some experiments, 5% Nu-Serum (Collaborative Research, Bedford, MA) was added after 7 days of growth. If gels had not contracted spontaneously, gel rims were loosened with a sterile needle about 12 hr before mounting in the Ussing chamber. Contraction was essential for development of a measurable transepithelial resistance.

Electrophysiologic Measurements. Monolayers were mounted lumen side up in a two-piece Plexiglas Ussing-type chamber and perfused on the luminal and serosal surfaces at a rate of 10 ml/hr with Earle's balanced salt solution (pH 7.4, 285 mOsm). The luminal bath was open so that cells could be observed by microscope and intracellular microelectrode impalements could be made. Solutions were gassed with 95% O_2-5% CO_2 and warmed by water jackets (32–37°C). Bipolar currents of $\pm 133 \times 10^{-6}$ A/cm^2 (2-sec duration, every 10 sec throughout the experiment) were passed between Ag-AgCl electrodes by means of a pulse generator and voltage clamp device (VCC 600; Physiologic Instruments, Houston, TX). The voltage responses were measured through two calomel electrodes, displayed digitally, and recorded. The chamber opening was 0.16 cm^2. Both luminal and serosal bathing solutions could be changed in about 3 min by valves.

Before mounting the epithelium, any voltage asymmetry of the calomel electrodes was offset and fluid resistance compensated. Collagen disks without confluent monolayers had no resistance. Where indicated, protamine was dissolved in the luminal bath (50 μg/ml). The protamine effect was reversed by heparin added to the luminal bath (0.4 units/μg protamine).

Cell membrane potentials were measured in monolayers bathed for 15–45 min in Earle's solution with 0.4 m*M* or 4.0 m*M* KCl using conventional microelectrodes filled with 0.5 *M* KCl connected to a high-impedence electrometer (model FD223; WPI, New Haven, CT) through Ag-AgCl half cells. Microelectrode resistances were 10–50 MΩ. One of two criteria for successful impalement was used: (i) an abrupt hyperpolarization maintained (±2 mV) for at least 1 min and a return to baseline when the microelectrode was withdrawn from the cell; or (ii) an initial deflection followed by gradual hyperpolarization to a stable value. In either case, impalements were deemed acceptable only if the initial and final baseline values differed by no more than 3 mV.

Morphologic Techniques. Cells were fixed in 2% glutaraldehyde buffered with 0.2 *M* phosphate. For thin-section electron microscopy, tissues were postfixed in 1% OsO_4 buffered in 0.1 *M* phosphate, dehydrated in graded concentrations of ethanol, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate. For freeze-fracture, tissue previously fixed in glutaraldehyde was treated in graded concentrations of glycerol up to 30% and frozen in Freon 22. After fracture (−100°C), replicas were shadowed with platinum and carbon in a Denton DFE-3 apparatus. Sections were examined in a Phillips 200 electron microscope.

Results

Culture. Electron micrographs of tubular segments prior to initial plating revealed columnar cells with extensive microvilli, pinocytotic vacuoles, and infolding of the basal membrane, all characteristic of rabbit proximal tubules *in vivo* (Fig. 1). Within 24 hr after the initial inoculation in hormone-supplemented DME/F-12 media, cells began to migrate and grow out of these tubular fragments. Monolayers began to form within 1–2 days, depending on the cell density of the initial tubular fragment inoculum. Complete confluence, as determined by the advent of a measurable transepithelial resistance, was noted within 7–10 days and persisted for an additional 15 days, the longest time tested. Cells survive for up to 50 days. Confluence appeared to be enhanced by the addition of 5% Nu-Serum. Fibroblast overgrowth was not seen in serum-free media or after the addition of Nu-Serum provided monolayers were used within a few days. Dome formation can be demonstrated in cells grown on plastic tissue culture dishes (1), but domes were not seen on collagen.

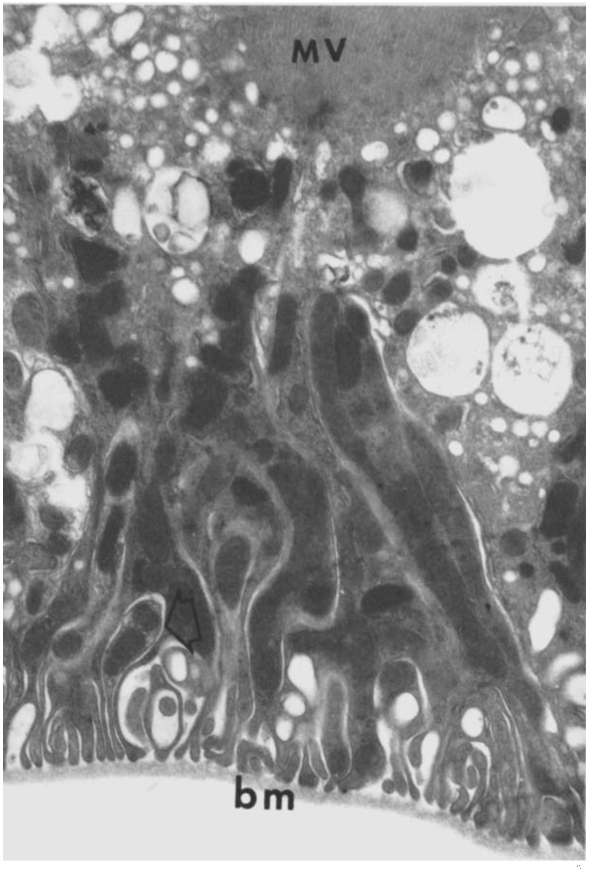

Figure 1. Section from proximal tubule segment prior to plating for culture. Note well-developed microvilli (MV), pinocytotic vacuoles, and moderate basal infoldings (arrow) characteristic of rabbit proximal tubule. The tubular basement membrane is labeled (bm) (original magnification ×19,425).

When cells were grown on Vitrogen disks, growth seemed accelerated when compared with cells grown on plastic tissue culture dishes. Between days 5 and 7, a concavity of the Vitrogen disk was observed as edges began to curl up and the disk started to shrink and wrinkle. Cells began to proliferate into small clusters or protuberances. These clusterings of cells seemed to occur on and around fragments of original tubular segments which were not removed during medium changes. Cells in the clusters had better developed microvilli and more extensive infoldings (Fig. 2) than adjacent cells growing in the flat portion of the monolayer (Fig. 3). Freeze-fracture of the apical segments of cells showed shallow tight junctions consisting in places of only one to two strands (Fig. 4), similar in appearance to tight junctional intramembranous structures observed in rabbit proximal tubules *in vivo* (14).

Electrophysiologic Measurements. Cell clustering was associated in time with the first appearance of nonzero transepithelial resistance and potential difference values, indicating that tight junction formation was now complete in the monolayer. This usually occurred by Day 7; all subsequent studies were done on 7- to 15-day-old cultures. At this time gels were contracted to 50–70% of their original diameter. In these cultures (Table I), the spontaneous transepithelial potential difference averaged +0.9 ± 0.3 mV with respect to serosa and the transepithelial resistance was 6.5 ± 0.5 $\Omega\ cm^2$ ($n = 8$). The range of measured transepithelial resistance was narrow, 4.5–9.2 $\Omega\ cm^2$, suggesting that confluence was complete in experiments in which resistance was nonzero. Luminal dilution potentials, in which half of the NaCl in the luminal bath was replaced isosmotically with sucrose, averaged −2.6 ± 0.2 mV ($P < 0.001$, $n = 12$), lumen negative with respect to serosa, indicating that this epithelium is anion selective. In about 20% of the monolayers studied after 7 days, no resistance could be measured. These cultures were discarded.

To determine whether the cell monolayers were capable of altering their resistance in response to an external stimulus, protamine (50 μg/ml) was applied to the luminal side and resulted in a 22 ± 3% increase in resistance over the baseline ($n = 8$). Resistance increments were reversed by heparin added to the luminal bath. Protamine significantly reduced the electroposi-

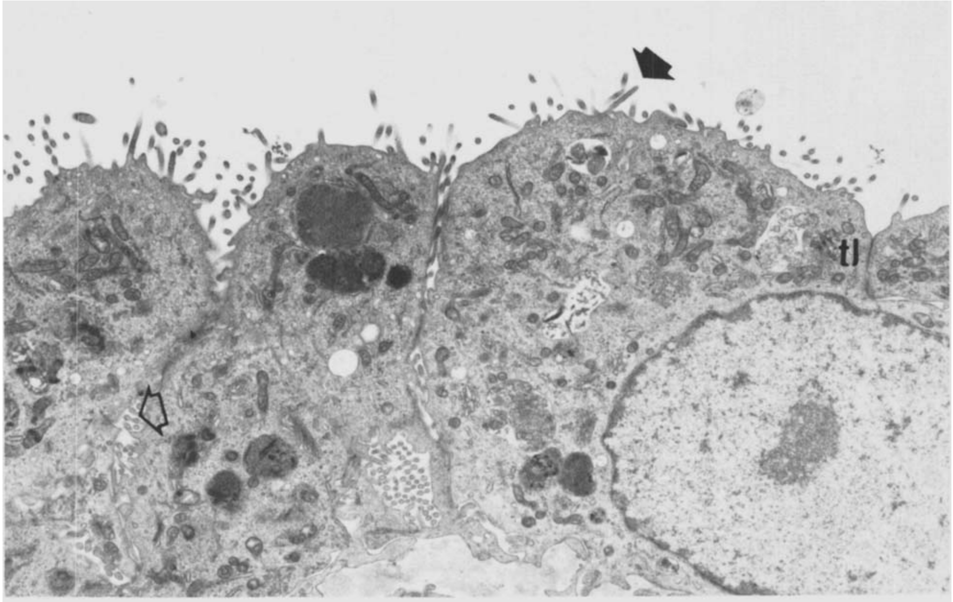

Figure 2. Monolayer cells from "tubular bud." Groups of cells can be seen extending upward from the monolayer after 7–10 days in culture, possibly in an attempt to differentiate into a tubular form. These cells show moderate numbers of microvilli (solid arrow), infoldings of the basolateral membrane (open arrow), and tight junctions (tj). In the adjacent cell, a tight junction is not identified except perhaps at the base where cell membranes are in close apposition (original magnification ×7,600).

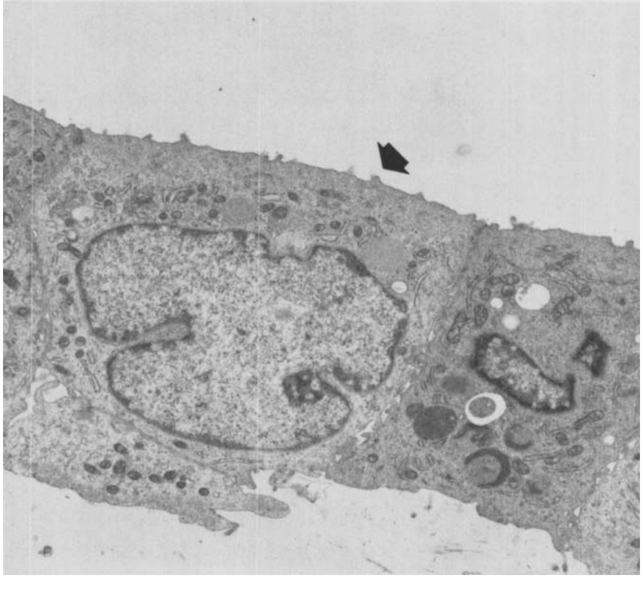

Figure 3. Adjacent cells located a short distance from cells in Figure 2 in flat portion of monolayer. Microvilli are rudimentary (arrow, compare with cells shown in Fig. 2) (original magnification ×7,600).

tive transepithelial potential difference ($P < 0.05$, $n = 8$). A typical response to protamine is shown in Figure 5. The peak response to protamine occurs within 10–15 min and the time course for heparin reversal is approximately the same.

Apical membrane potentials (Table II) recorded with microelectrodes averaged -49 ± 3 mV ($n = 22$) when the bathing solution contained 4 mM K. When external medium K^+ was decreased to 0.4 mM, the intracellular potential averaged -75 ± 4 mV ($n = 10$). Figure 6 shows one type of impalement that was seen, namely, hyperpolarization (after the initial deflection) to a stable value. This is usually interpreted as repair or resealing of the membrane around the electrode tip after the impalement. Tracings in the shape of square waves were also commonly seen.

Discussion

Epithelial monolayers have been successfully cultured from the proximal tubule cells of several species, including flounder (8), rat (7), rabbit (1, 6), and human (15, 16). The preparation of Chung *et al.* (1) has shown a number of functional characteristics of proximal tubules, including sodium-dependent glucose (11, 17) and phosphate (10) uptake and cAMP levels stimulated by parathyroid hormone but not by vasopressin or calcitonin (1). In addition, the production of prostaglandins has been investigated (12). As such, it is the best-characterized primary culture of proximal tubular cells currently under study. However, the electrophysiologic properties of this preparation have not been evaluated to determine whether monolayers are differentiated proximal tubular cells and whether measurements are comparable to those values obtained by micropuncture *in vivo* or *in vitro* in isolated perfused tubules. This was the object of the present experiments.

With the method of Chung *et al.* (1) proximal

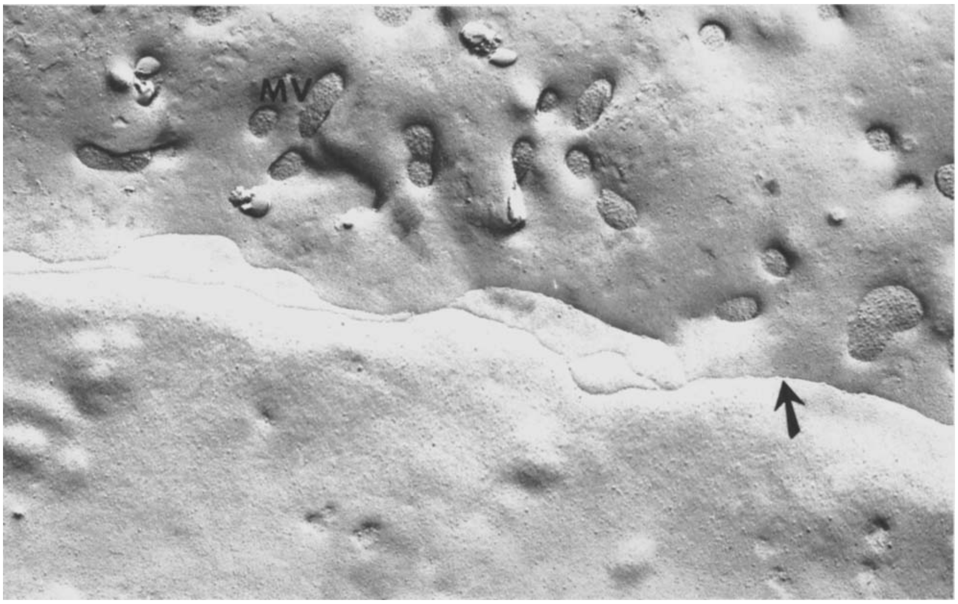

Figure 4. Freeze-fracture replica of tight junction from rabbit proximal tubular cell monolayer 10 days after initiating culture. Freeze-fracture reveals intramembranous structure of the tight junction which consists of one (arrow) or two strands (or grooves in EF face), typical of proximal tubular cells. Microvilli (MV) are sparse and are cut largely in cross-section (original magnification ×42,000).

Table I. Electrophysiologic Parameters Measured *In Vitro* in Monolayers of Cultured Rabbit Proximal Tubular Cells

Experiment	Transepithelial resistance (Ω cm^2)	Spontaneous potential difference (mV)[a]	Protamine response	
			Potential difference (Δ mV)	Resistance (Δ %)
1	6.6	+0.1	0	36
2	4.9	+0.9	−0.4	17
3	9.2	−0.1	−0.2	20
4	7.2	0	0	20
5	4.5	+2.8	−0.5	18
6	6.8	+1.0	−0.3	30
7	5.5	+1.7	−0.6	17
8	7.2	+0.9	0	15
Mean ± SEM	6.5 ± .5	+0.9 ± .3	−0.3 ± .1	22 ± 3
		$P < 0.05$	$P < 0.05$	$P < 0.001$

[a] Measured with respect to serosa.

tubule cells and tubular fragments are isolated by a simple sieving procedure and contaminating glomeruli are removed magnetically. We initially cultured these cells on Millipore filters as a substrate. Although it was evident under the light microscope that cell growth subsequently occurred, this procedure consistently yielded cultures in which the transmural potential difference and resistance were zero. We presume that this indicated a lack of total confluence or possibly the presence of cells that were not differentiated sufficiently to express polarized transport functions. Electron micrographs indicated that cells grown this way were flat, squamous in appearance, and lacked morphologic characteristics of mature proximal tubule cells.

We subsequently plated cells onto a flexible collagen substrate. One of the more interesting aspects of this study was the uniform finding that cultures had zero resistance until collagen gels were seen to contract spontaneously. Floating collagen gels have been found to promote expression of differentiated structure and function in other cultured cells, including hepatocytes (18) and the epithelia of flounder proximal tubules (8), the mammary gland (19–21), and the gastric mucosa (22). Each of these cell types was observed to contract the collagen gel and, in the case of mammary gland cells, the accompanying change in cell shape from squamous to cuboidal was essential for the appearance of differentiated function. This is thought to be a gen-

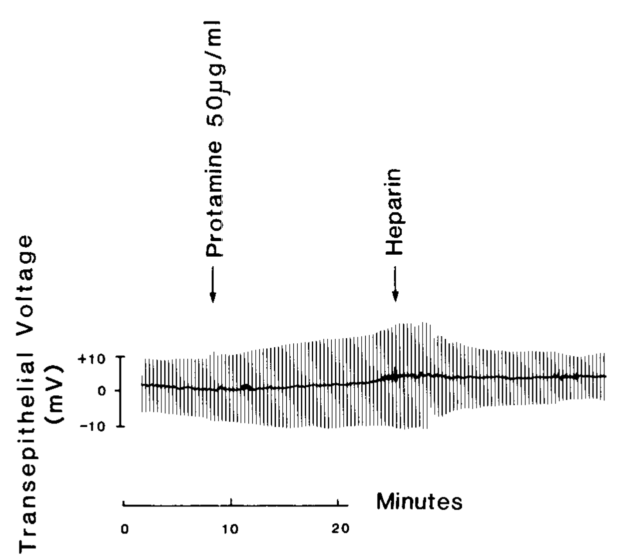

Figure 5. Typical electrophysiologic experiment demonstrating protamine response (reproduced from actual record). Transepithelial voltage is given on the ordinate. Note that shortly after protamine, 50 μg/ml, is perfused into the luminal compartment, bipolar voltage deflections begin to increase, leveling off in 10–12 min. Removal of the protamine-containing bath and replacement with a bath containing heparin reduces amplitude of deflections to baseline. Amplitude of voltage deflections are proportional to resistance since a constant current was passed (± 20 μA). Spontaneous transepithelial potential difference is measured when no current is passed. A change in this parameter would be reflected by an increase or decrease in midline voltage.

Table II. Apical Membrane Potentials in Rabbit Proximal Tubule Monolayer Cells

K^+ concentration of bathing medium (m*M*)	Voltage (mV)[a]
0.4	−75 ± 4 (10)[b]
4.0	−49 ± 3 (22)

[a] Measured with respect to luminal bath.
[b] Number of impalements given in parentheses.

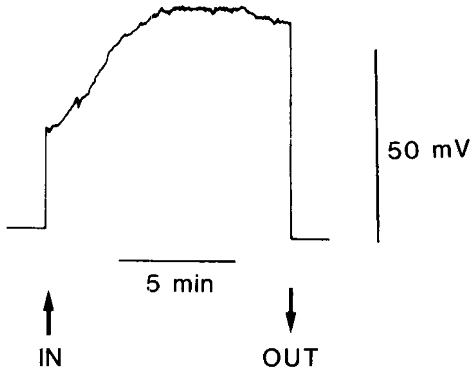

Figure 6. Microelectrode impalement of cultured rabbit proximal tubule cells. Conventional open tip electrodes were filled with 0.5 *M* KCl; those with resistances of 10–50 MΩ were used to determine apical membrane potentials. Tracing shows hyperpolarization to a stable value after the initial deflection. Impalements approximating square waves in shape were also seen.

eral feature of epithelial primary cultures. The mechanism for gel contraction is not apparent from our study, although it is possible that fibroblasts that penetrated the gel during the first few days could have initiated the event (23).

Handler and co-workers (24, 25) have emphasized the necessity of basolateral nutrition for the differentiation of epithelial cells. It is likely that the permeable collagen substrates used in our study also contributed to the growth, differentiation, and attainment of confluence by this mechanism.

Since the use of a collagen substrate represents a departure from the original Chung *et al.* (1) preparation, we obtained electron micrographs to evaluate the cell morphology. We observed that concomittant with gel contraction, cell clusters began to form which were more columnar and had more extensive development of microvilli. These findings suggested that cells were undergoing further differentiation. The observation that a measurable resistance could not be recorded until this time suggests that tight junctional sealing is also a function of some critical level of differentiation.

Although our monolayers have many characteristics of proximal rabbit tubules, it is apparent from the morphology of the individual cells that monolayer function has probably not reached the level of differentiation appreciated *in vivo*. However, all cells appeared to be the same basic cell type but precise morphologic classification is made uncertain because cells are not fully differentiated. Furthermore, transepithelial resistance was either 0 (about 20% of the experiments) or tightly clustered around 6.5 Ω cm^2 (note that the SEM is <8%, Table I), suggesting that when confluency was achieved, the ionic conductivity of the monolayer was uniform. This in turn suggests (but does not prove) that the population of cells was itself uniform. Also, it should be stressed that all studies done to date with the Chung preparation confirm that cells studied 7–10 days after plating possess functional characteristics of proximal tubules, as described above.

The present electrophysiologic studies add further evidence that cells are proximal tubular in origin. The transepithelial resistance (R_t) of 6.5 Ω cm^2 obtained here is comparable to the values of 5.5 Ω cm^2 measured in rats *in vivo* (26), 7 Ω cm^2 found in isolated perfused rabbit proximal tubules (27–29), and 7 Ω cm^2 reported by Bello-Reuss and Weber (6) in proximal tubular cell monolayers in which cells were harvested by centrifugation in Percoll. This low transepithelial resistance value is also consistent with the presence of shallow, one- to two-strand tight junctions, which we observed in the present study. The observation that R_t increased by an average of 22% after luminal surface exposure to protamine and that the change could be reversed when protamine was replaced by heparin indicates that this small value for R_t is not artifactual. We have observed the same effects of protamine and heparin on another leaky epithelium, the *Necturus* gallbladder, and have proposed that protamine, which is highly cationic at neutral pH (pI > 10), interacts with one or more anionic sites on the cell and subsequently modulates tight junctional structure and conductivity through an unknown mechanism (30, 31). In this model, heparin, which is highly anionic, reverses the effect either by scavenging residual free protamine directly or by displacing protamine from its interaction site on the cell membrane. The present results indicate that cultured monolayers of proximal tubule cells may respond in the same way.

When one half of the luminal bath NaCl is replaced isoosmotically by sucrose, the luminal compartment becomes electrically negative (average, −2.6 mV) with respect to the serosa. Such measurements are usually interpreted as being indicative of ion selectivity by tight junctions. In these monolayers, tight junctions are anion selective, as reported in isolated perfused proximal convoluted tubules of the rabbit (32).

Although an apical membrane potential difference of −49 mV in a 4 m*M* K bathing medium is comparatively lower than the −66 to −74 mV measured *in vivo* in rat proximal tubule cells (33, 34), it agrees with the −51 mV reported in isolated perfused rabbit proximal tubule cells (35) and with the −49 to −51 mV reported in cultured rabbit proximal tubular cells by Bello-Reuss and Weber (6). The 26 mV average hyperpolarization in apical membrane potential when the K in the bathing media is decreased to 0.4 m*M* indicates some ionic selectivity of the apical membrane, although not to the extent predicted by a perfect K-selective electrode.

The results of this study indicate that cells grown on a flexible porous collagen substrate and allowed to contract show superior phenotypic expression of differentiated epithelial characteristics when compared with cells grown on inflexible or impermeable surfaces. This is true in terms of both morphology and electrophysiologic properties. It would seem likely that at least some cellular metabolic processes might also be affected by the choice of substrate. Hence, the preparation described here, which shows more differentiated electrophysiologic as well as morphologic characteristics, would be a superior choice for future metabolic and transport studies of the proximal tubule.

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