Electrophysiological Study of Isolated Perfused Human Collecting Ducts

ION DEPENDENCY OF THE TRANSEPITHELIAL POTENTIAL DIFFERENCE

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ABSTRACT Cortical and outer medullary collecting duct segments were dissected from human kidneys and perfused in vitro. The transepithelial potential difference was measured and found to be lumen positive $+6.8 \pm 0.6$ mV ($n = 20$). This lumen-positive potential difference was inhibited by ouabain and furosemide but not by acetazolamide. Replacement of chloride in bath and perfusion fluids caused a reversible decrease of the potential difference to near zero. We conclude from these studies; (a) the lumen-positive potential difference is dependent upon the presence of chloride ion suggesting the existence of an active electrogenic chloride reabsorptive process in the human collecting duct and (b) it is possible to examine human renal physiology directly using in vitro microperfusion of tubule segments.

INTRODUCTION

Our present knowledge of human renal physiology is based on data derived from two major sources: (a) clearance studies in both humans and animals and (b) micropuncture and in vitro microperfusion of various nephron segments of animals. The latter sources, micropuncture and in vitro microperfusion, have not been applied directly to the human kidney for obvious reasons. Rather, the extensive amount of data derived from various animal species have been extrapolated to man. While there is no major objective reason to doubt that for the most part this extrapolation is valid, it seemed empirically important to examine the human nephron directly. The present study was designed to investigate the electrical properties of isolated perfused collecting ducts. The only previous

report of in vitro microperfusion of a human tubule was by Abramow and Dratwa (1) who described the response of a single fetal collecting duct to antidiuretic hormone.

METHODS

Segments of human collecting ducts, both cortical and medullary, were dissected from slices of human kidney. The kidneys were obtained from our transplant service. All kidneys were harvested for transplantation and initially flushed with a Ringer's solution. Subsequently the kidneys were handled in one of two ways. They were either perfused on the Waters pump (Waters Instruments, Inc., Rochester, Minn.) or they were maintained in iced Ringer's until dissection. If the kidney was perfused on the Waters pump, it was perfused with an artificial solution containing all the major electrolytes in similar concentrations to plasma and, in addition, glucose and small amounts of insulin.

When the kidney was not used for transplantation, it was maintained on the perfusion pump until slices were taken for dissection. The decision not to use a given kidney for transplantation was made by the transplant team of nephrologists and surgeons, and was based on four major criteria: anomalous vasculature making re-anastomosis technically impossible, inability to find an acceptable recipient with respect to immunological criteria, poor perfusion characteristics on the Waters pump, and damage to a portion of the kidney incurred during the harvesting procedure; i.e., infarction of a pole of the kidney or damage to a major vessel preventing re-anastomosis. (The University of Texas Health Science Center Human Research Committee has given permission to use harvested kidneys for research purposes if the transplant team concludes that the kidney is not suitable for transplantation). 2–3-mm thick slices were cut perpendicular to the surface of the kidney through both the cortex and medulla. These slices were transferred to a dissection dish containing a solution simulating ultrafiltrate of plasma and containing calf serum 5% by volume (for composition see Table I). The dissected collecting duct segments were perfused in a manner identical to that described previously for rabbit tubules (2). During dissection it was clear that four distinct straight segments could be identified on the

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TABLE I
Composition of Solutions Used

Solution	Control perfusate*	Chloride-free solution
	mM	
NaCl	105	—
NaHCO ₃	25	25
NaCH ₃ SO ₄	—	105
KCL	5	—
KHCO ₃	—	5
Na ₂ HPO ₄	2.3	2.3
Na acetate	10	10
MgSO ₄	1	1
CaCl ₂	1.8	—
Ca acetate	—	1.8
Glucose	8.3	8.3
Alanine	5	5

* Same solution used as control bath with addition of calf serum 5% vol/vol.

basis of size. These segments corresponded to those of the rabbit, both with respect to relative size and to subsequent morphology during the perfusion (magnification $\times 400$). The segment with the greatest diameter was the pars recta, with the collecting duct, thick ascending limb, and thin limbs following in decreasing order of diameter. Shown in Fig. 1 are photographs of both rabbit and human cortical collecting ducts during perfusion. As can be seen, they are quite similar in their light microscopic appearance. The collecting duct segments were initially perfused with a solution simulating ultrafiltrate of plasma and bathed with an identical solution containing calf serum 5% by volume (Table I). Tubules were perfused at varying rates but normally below 10 nl/min. The transepithelial potential difference (PD)¹ was recorded using a circuit previously detailed (3). In this circuit the PD is measured by a battery-operated Keithley model 602 (Keithley Instruments, Inc., Cleveland, Ohio) electrometer. The stability and accuracy of this system is excellent with voltage drift and accuracy of reading within ± 0.3 mV throughout the duration of the experiment. The PD was continuously recorded by a Rikadenki model B-261 multipen recorder (Rikadenki Kogyo Co., Tokyo). After the PD was recorded at room temperature, the bathing fluid was brought to 37°C by means of a heating coil in the bath chamber. The PD response to this increase in temperature was recorded. As soon as the PD obtained a stable value (10–30 min), various additional maneuvers were utilized to define the origin of the transtubular potential. These maneuvers included: (a) addition of 10 μ M ouabain to the bathing medium; (b) addition of 0.1 mM acetazolamide to the perfusion and bathing fluids; (c) addition of 10 μ M furosemide to perfusate; and (d) replacement of chloride by methyl sulfate in bath and perfusate, Table I. The tubules were exposed to the chloride-free solutions for 5 min or less; however, the PD was stable during this time interval. When more than one maneuver was applied to a given tubule, it was returned to control conditions before each new maneuver. In the experiments reported, both cortical and medullary collecting ducts were used. However, because of identical results in both segments, the experimental

results were pooled. This does not exclude the possibility of differences between these two regions of the human collecting duct with respect to other functional parameters. All data will be expressed as a mean and standard error of the mean. Statistical analysis was accomplished by use of the paired *t* test.

RESULTS

Control PD and response to 37°C. A total of 20 tubules from four kidneys was examined. At room temperature all 20 tubules exhibited an initial lumen-positive potential varying from +1 to +3 mV. When the bath temperature was raised to 37°C, all tubules, over a period of 2–3 min, exhibited an increase in the positive PD to $+6.8 \pm 0.6$ mV (see Fig. 2), and thereafter maintained a stable PD. This increase was reversible when the bath was allowed to cool again. The presence of a lumen-positive PD in the absence of any ion gradients for diffusion, and the increase in the magnitude of this PD with increasing temperature suggest the presence of a metabolically dependent “pump” either delivering positively charged species into the lumen or removing negatively charged species from the lumen. To characterize further the origin of this PD, various transport inhibitors were introduced into the lumen or bath.

Response to ouabain. When ouabain, 10 μ M, was placed in the bath, the PD rapidly decreased towards zero (Fig. 3). Maximal decrease was evident within 2–3 min. However, after washing the ouabain from the bath there was no recovery. The tubular morphology appeared to change in the presence of ouabain with the tubule becoming darker and appearing more granular. We have observed similar morphologic changes with ouabain in various isolated perfused rabbit nephron segments. The response of the PD to ouabain is additional evidence for an active mechanism generating the base-line positive potential.

Response to acetazolamide. As will be discussed in greater detail subsequently, a lumen-positive transepithelial potential has been observed in the rabbit cortical collecting duct (4, 5). Although this positive PD in the rabbit has several postulated mechanisms, it has been shown to be inhibited by acetazolamide (4, 5). Therefore, 0.1 mM acetazolamide was first placed in the bath. As can be seen in Fig. 4, there was no significant change in the PD. With these same tubules, 0.1 mM acetazolamide was also added to the perfusate, again without a significant PD change (Fig. 4). Therefore, there appears to be a difference between the rabbit and human collecting tubules with respect to the effect of acetazolamide on the positive PD.

Response to furosemide. Since the discovery of a lumen-positive potential in the rabbit thick ascending limb of Henle (TALH) and the elucidation of active

¹ Abbreviations used in this paper: PD, potential difference; TALH, thick ascending limb of Henle.

FIGURE 1 Photographs of isolated rabbit (A) and human (B) collecting ducts during perfusion demonstrating their similar microscopic appearance. One micrometer division = $4.2\ \mu\text{m}$. (Magnification $\times 400$.)

chloride reabsorption as the origin of this PD (6, 7), it follows that a similar mechanism may exist in any other nephron segment that manifests a positive PD. Furosemide has been shown to reversibly inhibit the positive PD and active chloride reabsorption in the rabbit TALH (8, 9). Therefore, with $10\ \mu\text{M}$ furosemide in the perfusate, the changes in PD were observed in the human collecting duct. As shown in Fig. 5, there was a significant decrease in the lumen-positive PD from $+6.8 \pm 0.6$ to $+2.2 \pm 0.7$ mV with re-

covery to $+7.2 \pm 1.6$ mV when furosemide was removed. Thus, the positive PD in the human collecting duct responds in a similar fashion to the rabbit TALH when furosemide is present in the luminal fluid. This similar response is indirect evidence for an active chloride reabsorptive mechanism in the human collecting duct. The reversible decrease of PD in response to furosemide, decrease of PD in response to ouabain, and reversible decrease in PD with respect to cooling are all indices of tubular viability.

FIGURE 2 Frequency distribution of transepithelial potential difference of isolated human collecting ducts at 37°C.

Response to chloride replacement in perfusate and bath. To further characterize the origin of the positive PD, the perfusion and bath fluids were replaced with new solutions that contained no chloride. As shown in Table I, sodium chloride was replaced by sodium methyl sulfate and calcium chloride by calcium acetate. Both the osmolalities and the pH of the new perfusate and bath were the same as control fluids (pH 7.4 after gassing with 95% O₂ and 5% CO₂). Shown in Fig. 6 is the response of the control lumen-positive PD to chloride substitution in bath and perfusate. A significant reversible decrease towards a zero PD was observed from $+6.3 \pm 0.1$ mV with recovery to $+6.2 \pm 1.1$ mV. This response was immediate. It should be noted that since the bath and perfusate are identical in composition, neither ionic diffusion gradients nor asymmetric liquid junction potentials

FIGURE 3 Effect of ouabain (10 μ M in bath) on the lumen-positive PD of isolated perfused human collecting ducts. C = control period; E = ouabain added to bath.

are introduced by the new perfusion and bath fluids. Therefore, the reversible decrease of the lumen-positive PD when chloride is absent from perfusate and bath is further evidence for the requirement of chloride in the generation of the lumen-positive PD.

DISCUSSION

The present studies are significant in two major ways. First, they represent the first direct observation of nonfetal human nephron function. Indeed, they show it is possible to examine directly certain functional characteristics of human nephron segments utilizing the technique of in vitro microperfusion. We therefore have a new powerful tool for investigating human renal physiology to extend and perhaps modify the data derived from clearance studies and animal experimentation.

The second major significant aspect of the present studies is the observation of a lumen-positive PD in the human collecting duct. When perfused and bathed with solutions similar to those used in the present study, rabbit collecting ducts have also been shown to exhibit positive PDs (4, 5). Stoner et al. (5) have shown that inhibition of sodium transport via

FIGURE 4 Effect of acetazolamide (0.1 mM in bath and perfusate) on the lumen-positive PD of isolated perfused human collecting ducts. C = control; E = acetazolamide added to bath and perfusate; R = recovery.

FIGURE 5 Effect of furosemide ($10 \mu\text{M}$ in perfusate) on the lumen-positive PD of isolated perfused human collecting ducts. C = control; E = furosemide added to bath; R = recovery.

ouabain, amiloride, or sodium replacement with choline causes the previous lumen-negative potential in the rabbit cortical collecting duct to become positive. In rabbit collecting tubules with base-line negative PDs, we have also found the development of a positive PD when sodium transport is inhibited (4). In addition, Gross et al. (10) have shown that the origin of the negative potential in the rabbit collecting tubules is mineralocorticoid-dependent sodium transport. Collecting tubules from rabbits fed a high salt diet, resulting in suppression of endogenous aldosterone, frequently have spontaneously positive potentials. The origin of the lumen-positive PD in the rabbit collecting tubules has at least three possible sources: potassium secretion, hydrogen secretion, and chloride reabsorption. These three possible mechanisms apply equally well to the human collecting duct.

All human collecting ducts in the present study had base-line lumen-positive PDs which increased in magnitude when the bath temperature was raised to 37°C . One initial concern was whether perfusion on the Waters pump with artificial perfusate introduced a nonspecific artifact even though the tubules appeared morphologically normal. Two lines of evidence support that no artifact was present: (a) tubules dissected from kidneys harvested and not perfused on the Waters pump but immediately placed in iced Ringer's before dissection also had positive PDs, and (b) pars recta or straight segments of the proximal tubule from perfused kidneys exhibited negative PDs of -2 to -3

mV (unpublished observations) similar to the results found in the rabbit straight segments (11). We have subsequently perfused collecting ducts from several more human kidneys which had been perfused for a time on the Waters pump and also just placed in iced Ringer's. None of these collecting ducts exhibited a negative PD. The inability to observe a negative PD in the human collecting ducts has several possible explanations: (a) the absence of electrogenic sodium transport either dependent or independent of mineralocorticoid; (b) the capacity for mineralocorticoid-dependent sodium transport requiring mineralocorticoid but the absence of mineralocorticoid in our preparation; and (c) the capacity for electrogenic sodium transport independent of mineralocorticoid but somehow lost before our *in vitro* study. The present set of studies does not allow us to settle the question of whether electrogenic sodium transport exists in the human collecting duct.

The ouabain sensitivity of the lumen-positive potential suggests an active process underlying the generation of this PD. However, the response to ouabain is not specific with respect to defining the exact origin of the potential. Ouabain has been shown to inhibit active chloride transport in rabbit TALH (6, 8) and rabbit oviduct (12). However, in contrast to the findings in human collecting ducts, we have not found that ouabain inhibits the lumen-positive PD in rabbit collecting ducts. We have recently demonstrated in the rabbit cortical collecting duct the chloride dependence of the lumen-positive potential (4). In comparing the rabbit to human collecting ducts, we postulate that the former has a ouabain-

FIGURE 6 Effect of chloride replacement (methyl sulfate) in bath and perfusate on the lumen-positive PD of isolated perfused human collecting ducts. C = control; E = methyl sulfate replacement of chloride in bath and perfusate; R = recovery.

insensitive chloride pump while the latter has a ouabain-sensitive chloride pump. There are precedents for two kinds of chloride pumps. Nellans et al. (13) have proposed the existence of two separate chloride transport processes in rabbit ileum as have Alvarado et al. (14) and Watlington et al. in frog skin (15). The latter authors have found that these chloride pumps may be distinguished on the basis of the response to ouabain and acetazolamide (15).

While the experiments with acetazolamide in the perfusate and bath show no effect on the human collecting duct PD, an effect of acetazolamide has been observed in rabbit collecting ducts (4, 5). In rabbits this drug increases the magnitude of a negative PD or decreases the magnitude of a positive PD. While others have postulated that acetazolamide inhibits carbonic anhydrase-dependent electrogenic hydrogen secretion in the rabbit (5), in another paper we discuss the possibility of acetazolamide inhibition of active chloride reabsorption in the rabbit collecting duct (4). With respect to its effect on the PD, acetazolamide thus appears to produce different responses in human and rabbit tubules. Previous studies in several species have documented both the presence and absence of acetazolamide-induced changes in chloride transport. Acetazolamide has been shown to inhibit active chloride transport in rabbit ileum (16), frog skin (14, 15) frog cornea (17), and turtle urinary bladder (18), while it does not inhibit active chloride transport in rainbow trout gills (19).

The reversible decrease in the lumen-positive PD when furosemide is present in the perfusate provides strong evidence for active chloride reabsorption. Furosemide has been shown to inhibit the positive PD and lumen-to-bath transport of chloride in the rabbit TALH (8, 9). It is unlikely that the observed effect of furosemide is due to carbonic anhydrase inhibition since acetazolamide had no effect in either lumen or bath.

Finally, the replacement of chloride with methyl sulfate in bath and perfusate decreased the PD to zero. This response was immediate and reversible. This type of replacement experiment provides the strongest evidence that the lumen-positive PD in this segment is totally dependent upon the presence of chloride ion. The replacement of chloride on both sides of the epithelium excludes the introduction of both diffusion potentials and asymmetric liquid junction potentials as explanations for the PD changes. Alternative explanations for the observed PD change with chloride replacement include three major possibilities. First, the absence of chloride may nonspecifically affect the viability of the tubule and inhibit all transport phenomena. Although the short duration of chloride absence and rapid reversibility of the PD change argue against this, stronger evidence against

such an explanation lies in studies where we have used similar chloride replacement. In straight segments of proximal tubules of rabbits, chloride replacement with methyl sulfate or cyclamate did not affect tubule viability or base-line negative transport PD (11). Indeed, chloride replacement in the rabbit collecting duct which has a base-line negative PD caused the PD to become more negative, presumably secondary to decreased shunting of the sodium transport PD (4). The rabbit collecting duct therefore remains viable and still maintains a negative sodium transport PD in the absence of chloride. A second possible explanation for the effect of chloride replacement is the inhibition of a chloride-dependent process generating a PD by the transport of another ion. This alternative ion transport mechanism would most likely be hydrogen or potassium transport into the lumen. The lack of an effect of acetazolamide and the absence of any evidence for such a chloride dependent transport in other epithelia make the second alternative explanation very unlikely. The third possible explanation for the effect of chloride replacement is inhibition of a non one-to-one anion exchange process in which chloride is one of the anions (20). An example would be chloride reabsorption coupled to bicarbonate secretion with the chloride reabsorption being quantitatively greater. While such a mechanism may exist in other epithelia (21), it is unlikely, although untested, that it exists in human collecting ducts especially in light of the fact that acetazolamide does not affect the positive PD.

In summary, the effects of temperature, ouabain, acetazolamide, furosemide, and chloride replacement on the PD of in vitro perfused human collecting ducts were observed. The human collecting duct exhibits a lumen-positive PD inhibited by ouabain but not by acetazolamide. This PD is decreased reversibly by furosemide in the perfusate and by chloride replacement in perfusate and bath. These electrophysiological studies suggest the presence of active chloride reabsorption in the human collecting duct. However, it must be pointed out that the quantitative significance of the described chloride pump cannot be ascertained without unidirectional flux measurements. In addition, these studies indicate that important physiological data can be obtained directly from human nephron segments utilizing the technique of in vitro microperfusion.

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