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Urea Transport in Freshly Isolated and Cultured Cells from Rat Inner Medullary Collecting Duct

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Summary. Regulation of urea transport by vasopressin in inner medullary collecting duct (IMCD) cells is thought to be important for the urinary concentrating mechanism. Isolated tubule perfusion studies suggest the existence of a saturable urea carrier. We have measured ^{14}C-urea efflux in IMCD cells which were freshly isolated and grown in primary culture. Cells were isolated from rat papilla by collagenase digestion and hypotonic shock. In suspended cells, ^{14}C-urea efflux (J_{urea}) from loaded cells was exponential with time constant 59 ± 3 sec (SEM, n = 6, 23°C). J_{urea} had an activation energy of 4.1 kcal/mole and was inhibited 42 ± 7% by 0.25 mM phloretin and 30–40% by the high affinity urea analogues dimethylurea and phenylurea. J_{urea} was increased 40–60% by addition of vasopressin (10^{-8} M) or 8-bromo-cAMP (1 mM); stimulated J_{urea} was inhibited 55 ± 8% by the kinase A inhibitor H-8. Phorbol esters and epidermal growth factor did not alter J_{urea}. IMCD cells grown in primary culture were homogeneous in appearance with >fivefold stimulation of cAMP by vasopressin. The exponential time constant for urea efflux was 610 ± 20 sec (n = 3). J_{urea} was not altered by vasopressin, cAMP or phloretin. Another function of in vivo IMCD cells, vasopressin-dependent formation of endosomes containing water channels, was absent in the cultured cells. These results demonstrate presence of a urea transporter on suspended IMCD cells which is activated by cAMP and inhibited by phloretin and urea analogues. The urea transporter and its regulation by cAMP, and cAMP-dependent apical membrane endocytosis, are lost after growth in primary culture.

Key Words urea · kidney collecting duct · vasopressin · cAMP · phloretin · cell culture

Introduction

Urea transport in the inner medullary collecting duct (IMCD) of mammalian kidney and in the amphibian urinary bladder is regulated by vasopressin acting through a cAMP-dependent pathway (Star et al., 1988). Although water transport is regulated by vasopressin in these tissues by a membrane cycling mechanism (Brown, 1989; Verkman, 1989), the transport pathways and regulatory mechanisms for water and urea transport are probably different (Knepper, Sands & Chou, 1989; Shi, Brown & Verkman, 1990). Recent evidence obtained by perfusion of isolated tubule segments suggests that urea transport in the vasopressin-stimulated medullary collecting duct is mediated by a saturable carrier mechanism (Chou & Knepper, 1989) which is similar to that of red blood cells (Macey, 1984). It is not known whether urea carriers are inserted into and retrieved from plasma membranes, or whether they are regulated by in situ post-translational modification.

The development of isolated cell and cell culture models for regulation of urea transport is an important goal for further biophysical and biochemical analysis of the kidney urea transporter. Techniques for the rapid isolation of purified cells from IMCD have been developed recently (Grenier, Rollins & Smith, 1981; Teitelbaum & Berl, 1986). The suspended cells were viable and suitable for studies of membrane transport (Stokes, Grupp & Kinnes, 1987; Grupp et al., 1989) and for growth in primary culture (Teitelbaum & Berl, 1986; Husted, Hayashi & Stokes, 1988). The intracellular signaling and pH regulatory pathways of cells grown in primary culture have been characterized (Nord & Hart, 1989; Teitelbaum & Strasheim, 1989); however, their urea and water transport properties have not been studied.

We report here the urea transport characteristics of IMCD cells that have been freshly isolated and grown in primary culture. The main purpose of this study was to evaluate the isolated cell and primary culture models for identification of urea transporters and for studies of its regulation. Evaluation of the cell culture model is particularly important because it has been used in biochemical studies without evidence that the major physiological activities of in vivo IMCD cells, vasopressin-dependent urea and

water transport, remain functional in the cultured cells. A second important purpose of this study was to evaluate the suitability of fresh tissue and cultured cells as a source of mRNA for expression cloning of kidney urea and water transporters.

Materials and Methods

Cell Preparations

IMCD cells were obtained by a modification of the methods of Grenier et al. (1981) and Teitelbaum and Berl (1986). Female Sprague-Dawley rats (150–200 mg) were anesthetized with ether and sacrificed by cervical dislocation. Kidneys were removed immediately and placed in ice-cold Krebs-Ringers buffer containing (in mM): NaCl 128, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1.2, $NaCH_3COOH$ 10, NaH_2PO_4 2, glucose 10, Tris 10, pH 7.40. For cell culture, 100 U/ml penicillin and 100 μg/ml streptomycin were added. Kidneys (~750 mg/kidney) were bisected and the white portion of the papilla was removed (~40 mg/kidney). In some experiments, the papilla was further divided into outer and inner portions. The papilla was diced with a razor blade into very small pieces. The diced papilla was digested in 0.2% collagense (Type 1, Sigma; 1.25 ml per papilla) at 37°C in a 5% CO_2/95% air incubator for 45 min. 0.001% DNAse was added to minimize cell clumping and the suspension was incubated for an additional 45 min. The suspension was shaken and drawn through a plastic pipette 10 times every 15 min until there were no visible cell clumps. To lyse non-IMCD cells (Teitelbaum & Berl, 1986), two volumes of distilled water were added rapidly to the cell suspension. The suspension was centrifuged at 200 × *g* for 5 min to obtain a cell pellet. The pellet consisted of a few single round cells with diameter 8–10 μm with many cell clumps and short tubule fragments; >90% of cells excluded the vital stain Trypan blue.

For cell culture, the same procedure was used except that the DNAse addition was omitted. The cell pellet was suspended in Krebs-Ringer's buffer containing 10% bovine serum albumin and centrifuged at 50 × *g* for 3 min to yield a final cell pellet. The pellet was suspended in 90% Ham F-12 and 10% Liebovitz L-15 medium containing 10% fetal bovine serum, 5 μg/ml insulin, 5 pM triiodothyronine, 50 nM hydrocortisone, 25 ng/ml PGE_1, 30 nM spermine and 250 nM spermidine, and plated on 25-mm plastic dishes. Generally three rat kidneys were used to seed one 12-well dish. After 24 hr the fetal bovine serum concentration was reduced to 1%. The medium, referred to subsequently as "growth medium," was changed on day 3 and cells were used on days 4–5 unless stated otherwise. Cells at this time were fairly homogeneous in appearance with visible dome formation, similar to morphology reported by Teitelbaum and Berl (1986). There was ~50 μg of cell protein per dish.

Baseline and vasopressin-stimulated cAMP formation was measured at 37°C using a radioimmunoassay kit from New England Nuclear. After incubation of IMCD cells with methyl-isobutyl xanthine (IBMX) and vasopressin for 10 min, 0.01 M HCl was added to release intracellular cAMP. cAMP content was 3 ± 1 fm/μg protein in the absence of IBMX and vasopressin, 60 ± 10 fm/μg protein in the presence of 0.2 mM IBMX, and 298 ± 30 fm/μg protein in the presence of 0.2 mM IBMX and 10^{-8} M vasopressin. These results are in agreement with previous data. Fura-2 measurements of intracellular free calcium showed that neither vasopressin (10^{-11} to 10^{-8} M) nor bradykinin (10^{-8} M) caused a transient elevation in calcium, indicating no significant fibroblast contamination in the cell culture.

For comparison with results in IMCD cells, the established kidney epithelial cell lines LLC-PK1 and MDCK, and the toad epithelial cell line A6 were used. MDCK cells (ATCC CCL 34) and LLC-PK1 cells (ATCC CCL-101) were grown to confluency at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and used between passages 61–70 (MDCK) and 221–240 (LLC-PK1). A6 cells (ATCC CCL-102) were grown at 26°C in a 5% CO_2 incubator in Eagle's medium and used between passages 59–70.

Urea Transport Measurements

Urea efflux was measured using ^{14}C-urea (New England Nuclear, Bedford, MA, 58 mCi/mmol). For the freshly prepared cells, the pellet was suspended in the culture medium (5 ml per kidney papilla) and incubated for 60 min at 37°C for metabolic stabilization. Cells from two kidney papillas were pelleted at 200 × *g* for 5 min and suspended in 100 μl of growth medium containing 6 μCi ^{14}C-urea. After a 10-min incubation at 23°C to load cells with urea, the suspension was diluted 100-fold with growth media and stirred slowly at specified temperature. In some experiments, activators or inhibitors were added before and/or after the dilution. To measure urea efflux, 0.5 ml of the cell suspension was removed at a series of time points and filtered on 1.2-μm HAWP Millipore filters prewetted with wash solution (Hanks buffer containing 25 mM urea). The filter was washed three times with 3 ml of ice-cold wash solution. The washing was completed in under 5 sec. The filter was dissolved with 7 ml scintillation fluid (Beckman, Fullerton, CA) and radioactivity was counted on a scintillation counter. In some experiments, a double-label technique was used in which ^{3}H-O-methyl-glucose served as a cell volume marker (*see* Results).

To measure urea efflux from cultured cells, 2 μCi ^{14}C-urea was added to 0.5 ml of medium covering one 25-mm diameter plastic dish. Cells were incubated for 90 min at 37°C and washed four times with 2 ml of growth medium not containing urea. Urea efflux was initiated by adding 1 ml of growth medium to the washed cells. In some experiments, activators or inhibitors were added. The culture dish was shaken continuously in a temperature-controlled environment. At specified times, 0.1-ml aliquots of the growth medium were removed and dissolved in scintillation fluid for measurement of ^{14}C-urea radioactivity. The ^{14}C-urea remaining in the cells at the end of the experiment was measured by dissolving the adherent cells with 0.1 M NaOH.

Urea Permeability Calculations

Because the external volume was much greater than the intracellular volume in ^{14}C-urea efflux measurements, cell radioactivity, $R(t)$, was fitted to a single-exponential function,

$$R(t) = A_1 \exp(-t/t_{urea}) + A_2 \quad (1)$$

where A_1 is the intracellular radioactivity (in dpm) at 0 time, t_{urea} is the exponential time constant and A_2 is background radioactivity at infinite time. It is assumed in Eq. (1) that cells loaded with urea have homogeneous geometry and membrane urea permeability. The urea permeability coefficient, P_{urea} (in cm/sec), is related to t_{urea} by

$$P_{urea} = [(S/V)t_{urea}]^{-1} \quad (2)$$

where S/V is cell surface-to-volume ratio. $R(t)$ was calculated from measured dpm in isolated cell experiments and from supernatant and cell dpm for cultured cell experiments; data were plotted as relative cell radioactivity, determined by normalization of $R(t)$ to unity at 0 time after subtraction of background radioactivity.

Osmotic Water Transport in Endosomes

Apical endosomes were labeled with 6-carboxyfluorescein (6CF) in intact cells by fluid-phase endocytosis as described previously (Verkman et al., 1988; Shi & Verkman, 1989). Cells grown on 12-well dishes were incubated in growth medium containing 15 mM 6CF (pH 7.4) for 30 min at 37°C. In some experiments vasopressin or 8-Br-cAMP was added before and/or after addition of 6CF. Cells from one 12-well dish containing 6CF-labeled endosomes were washed six times with 5 ml of ice-cold growth medium, scraped, and homogenized in buffer A (50 mM mannitol, 5 mM Na phosphate, pH 8.5) using a Potter-Elvehjem homogenizer. All procedures were performed at 0–4°C. The suspension was centrifuged at 2,500 × g for 10 min to remove heavy cellular debris. The supernatant was centrifuged at 100,000 × g for 45 min to obtain a microsomal pellet containing endosomes labeled with 6CF. The microsomal pellet was washed once in buffer A to remove external 6CF. The final microsomal pellet was homogenized with a 25-gauge needle and suspended at ~0.5 mg protein/ml.

Endosome osmotic water permeability (P_f) was measured by a stopped-flow fluorescence quenching technique. The endosome suspension was subjected to a 60-mOsm inwardly directed sucrose gradient in a Hi-Tech SF51 stopped-flow apparatus (Wiltshire, England). The osmotic gradient caused water efflux and a decrease in endosome volume, resulting in an instantaneous decrease in 6CF fluorescence due to self-quenching. There was no influence of nonfluorescent vesicles on the fluorescence signal. Fluorescence was excited at 465 ± 5 nm and monitored at >515 nm. The instrument dead time was 1.7 msec and the mixing and electronic response times were under 1 msec. P_f was calculated from the time course of fluorescence as described previously (Chen, Pearce & Verkman, 1988).

Osmotic Water Transport in Isolated Cells

Osmotic water permeability of isolated IMCD cells was measured by a stopped-flow light-scattering technique as described by Meyer and Verkman (1986). A suspension of isolated cells in Krebs-Ringer's buffer (~2 × 10^7 cells/ml) were subjected to a 300-mOsm inwardly directed gradient of sucrose to shrink cells by 50%. The time course of 90° scattered light intensity at 520 nm was measured. To estimate urea permeability, sucrose was replaced by urea (*see* Results).

Results

The time course of ^{14}C-urea efflux in freshly isolated suspended cells is shown in Fig. 1. The data fitted well to a single-exponential function. There was relatively little biological variability in urea transport in our cell preparation; in six separate cell preparations, the exponential time constants measured in the absence of inhibitors or activators at 23°C were (in sec): 50, 65, 61, 70, 50 and 58, giving an average value of 59 ± 3 sec (SE). The time constant was temperature dependent, decreasing from 86 ± 5 sec at 10°C to 46 ± 4 sec at 37°C. The data at 10, 23 and 37°C give an apparent activation for urea transport of 4.1 kcal/mol.

The effects of several possible inhibitors of urea transport were examined. In the red blood cell, urea permeability is strongly inhibited by phloretin and lipophilic urea analogues (Mayrand & Levitt, 1983; Macey, 1984). Figure 1 shows data from single experiments and Fig. 3 summarizes paired data from a series of separate experiments. Both phloretin and the high affinity urea analogue phenylurea inhibit urea transport. In five sets of paired experiments, 0.25 mM phloretin inhibited urea transport by 42 ± 7%. Urea transport was inhibited 33 ± 6% by 10 mM dimethylurea and 37 ± 7% by 10 mM phenylurea. These compounds do not alter the urea permeability of phosphatidylcholine liposomes measured by the light-scattering technique of Chen and Verkman (1987) (*data not shown*).

Urea transport was measured in response to activation of the cAMP pathway by vasopressin and 8-Br-cAMP (Figs. 2 and 3). Both compounds increased urea efflux by ~50%. The selective protein kinase A inhibitor H-8 (O'Grady et al., 1988) inhibited 8-Br-cAMP activated urea permeability by 55 ± 8% (n = 3). The protein kinase C activator PMA and the phospholipase C activator EGF did not alter urea permeability, nor did PMA affect the stimulation of urea permeability by 8-Br-cAMP. These data indicate that urea permeability in the freshly isolated IMCD cells is regulated by the cAMP pathway.

Several lines of evidence indicate the urea transport under control conditions is in part mediated by a cAMP-dependent carrier. Urea transport was inhibited to similar extents by H-8, phloretin and the high affinity urea analogues. Addition of H-8 to phloretin-inhibited cells caused no further inhibition of urea transport.

Two types of experiments were performed to determine whether there was very rapid urea transport in a subpopulation of cells that was not observed because of the limited time resolution of the ^{14}C-urea efflux assay. In the first experiment, cells were labeled to equilibrium (60 min) with ^{14}C-urea and the slowly transported volume marker ^{3}H-O-methyl glucose. The ratio of ^{14}C/^{3}H counts (R) was measured in the supernatant and at 5 sec after dilution with nonradioactive buffer and filtration. In three sets of experiments, $R/R_{supernatant}$ was 0.78 ± 0.06, indicating that very fast urea transport may be pres-

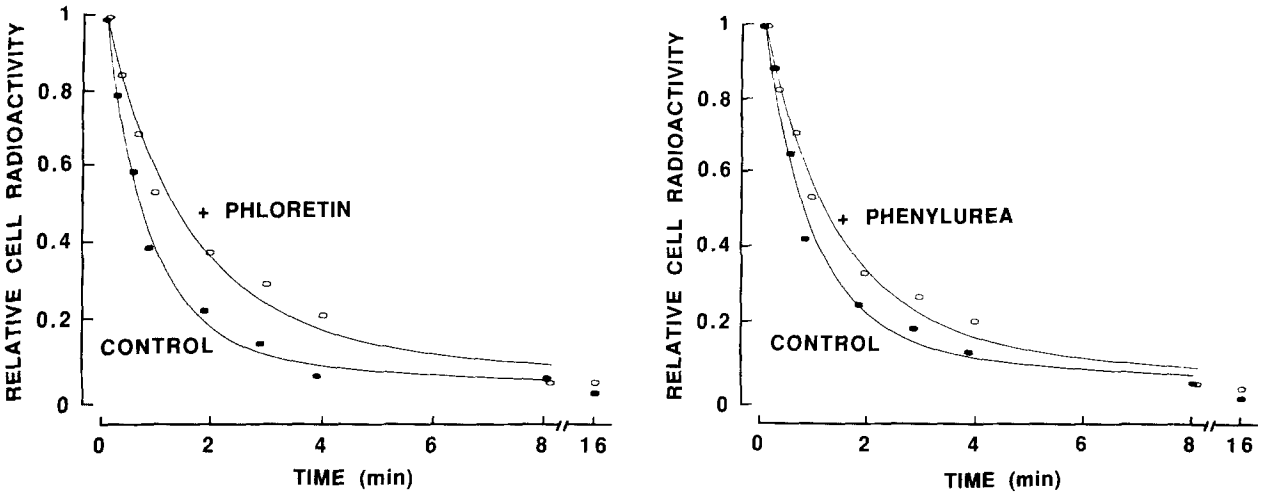

Fig. 1. Effect of inhibitors on ^{14}C-urea efflux in freshly isolated IMCD cells. ^{14}C-urea efflux was measured upon 100-fold dilution of cells loaded with ^{14}C-urea as described in Materials and Methods. Measurements were carried out at 23°C. Inhibitors were present only in the efflux buffer. Paired experiments are shown in which cells were divided equally into control and inhibitor groups. Data were normalized to unity at zero time and fitted to the single-exponential function in Eq. (1). Inhibitor concentrations were 0.25 mM (phloretin) and 10 mM (phenylurea). Results from a series of paired experiments are given in Fig. 3

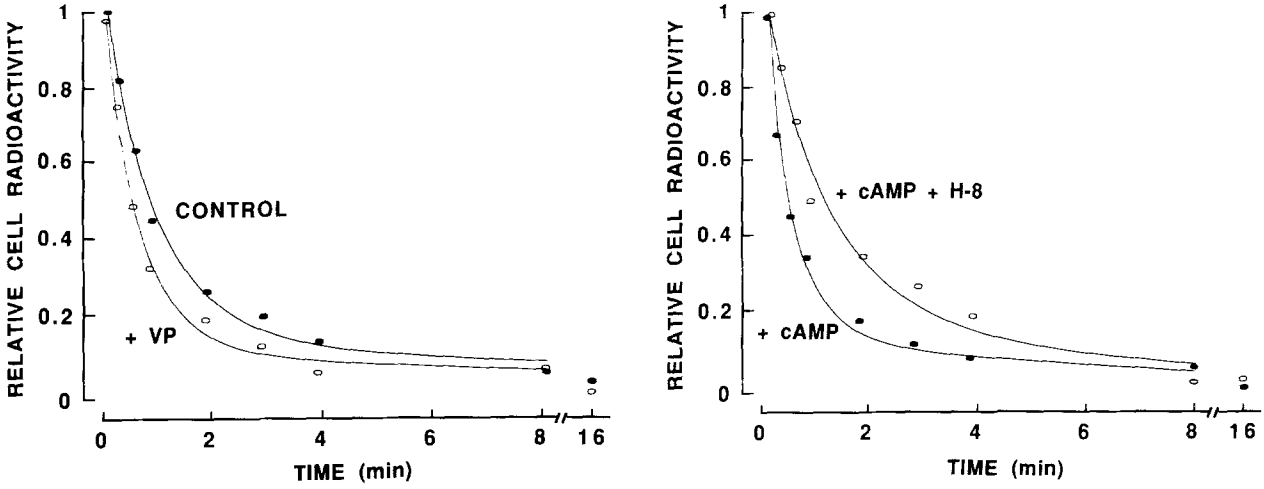

Fig. 2. Effects of cAMP effectors on ^{14}C-urea efflux in freshly isolated IMCD cells. ^{14}C-urea efflux was measured as in Fig. 1. Cells were incubated with effectors (vasopressin 10^{-8} M, 8-Br-cAMP 1 mM, H-8 0.2 mM) for 10 min prior to the 100-fold dilution into cold buffer. Effectors were also present in the efflux buffer. Paired experiments performed at 23°C are shown with single-exponential fits. Results from a series of paired experiments are given in Fig. 3

ent in a subpopulation of cells comprising <25% of the total cell volume.

In a second type of experiment, rapid urea transport was searched for by a stopped-flow light-scattering technique (Fig. 4). Suspended IMCD cells were subjected to 300-mM inwardly directed gradients of the impermeant solute sucrose, or urea. In response to the sucrose gradient, there was a rapid increase in scattered light intensity giving an osmotic water permeability (P_f) of 0.02 cm/sec. P_f was inhibited by ~70% by 0.5 mM $HgCl_2$. In response to the urea gradient, there was a rapid increase in scattered light intensity due to osmotic water efflux, followed by a slow, almost linear decrease in scattering over 30 sec due to urea influx. The absence of a rapid decrease in scattering suggests absence of a population of cells with urea efflux time constant of under 30 sec. However, because the light-scattering properties of different cell types are not known, it is not possible to establish a quantitative upper limit to the cell fraction with fast urea transport. The light-scattering technique could not be used to study urea

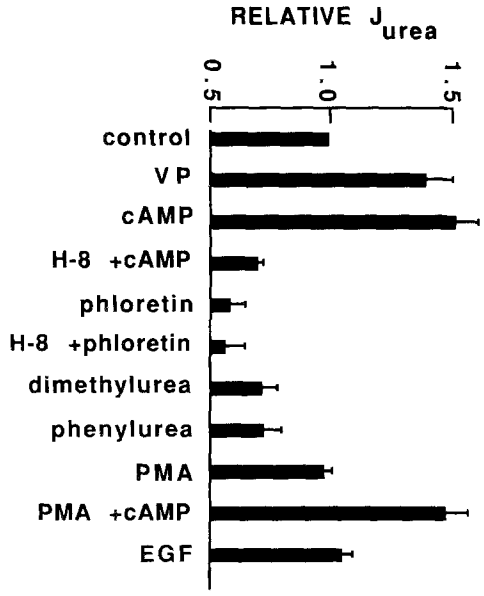

Fig. 3. Regulation of urea transport in freshly isolated IMCD cells. Data are summarized for 3–7 separate sets of measurements. Data are the mean ± SE for paired measurements. Concentrations were 10^{-8} M (VP), 1 mM (8-Br-cAMP), 0.2 mM (H-8), 0.25 mM (phloretin), 10 mM (dimethylurea), 10 mM (phenylurea), 1 μM (PMA), 1 μM (EGF). Compared to the control, all compounds produced significant effects ($P < 0.01$) except for PMA and EGF. Relative J_{urea} for VP, cAMP and PMA + cAMP were not significantly different

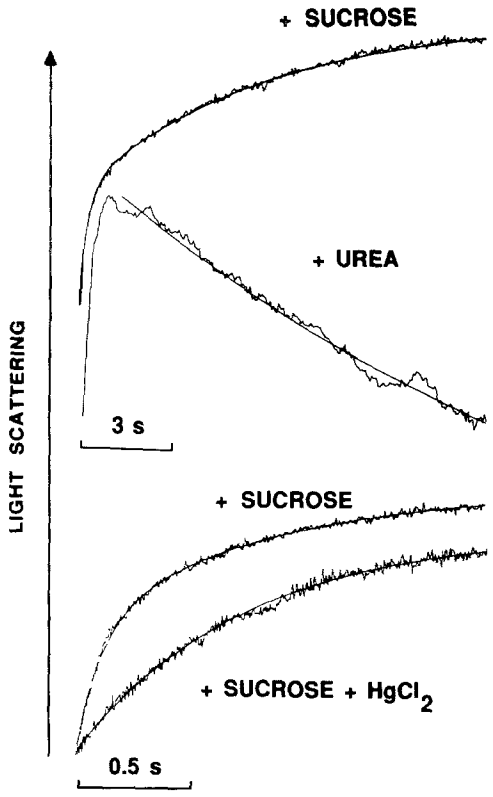

Fig. 4. Osmotic water and urea transport in freshly isolated IMCD cells measured by a stopped-flow light-scattering technique. Suspended IMCD cells were subjected to 300-mM inwardly directed gradients of sucrose and urea as described in Materials and Methods. Osmotic water efflux caused a decrease in cell volume and an increase in scattered light intensity; urea influx caused an increase in cell volume. The fitted curves for sucrose gradient data were biexponential with maximum P_f of 0.02 cm/sec (control)

transport over times longer than 30 sec because of cell settling in the apparatus.

Figure 5 shows the effects of phloretin and cAMP agonists on urea efflux in the IMCD cells grown in primary culture. In three separate sets of cell cultures, the exponential time constant for urea efflux was 610 ± 20 sec (SE) under control conditions. In three sets of paired experiments, 250 μM phloretin changed the urea efflux rate by −4 ± 7%. 10^{-8} M vasopressin and 1 mM 8-Br-cAMP changed urea efflux by 3 ± 6% and 0 ± 4%, respectively. The slow urea efflux and lack of effect of cAMP agonists and phloretin results suggest that carrier-mediated urea transport is absent in the cultured cells.

Several modifications in the transport assay protocol and cell growth conditions were made to investigate whether the urea transport properties of the in vivo and freshly isolated IMCD cells could be maintained in the cultured cells. Cells were incubated with 1 mM 8-Br-cAMP during and for 60, 10 or 0 min before the urea efflux assay. Urea transport did not change. There was no effect of 8-Br-cAMP when urea transport was assayed at 23 instead of at 37°C. There was no effect of studying cells after eight days of growth. Finally, addition of 10^{-11} or 10^{-8} M vasopressin to the cell culture medium for 24 hr before urea transport measurements did not affect the urea flux.

Another important property of IMCD cells in vivo is vasopressin-stimulated water permeability. Vasopressin has been shown to stimulate the endocytosis of functional water channels in the papilla of Brattleboro rats (Verkman et al., 1988). Endosomes were labeled in intact cells by fluid-phase entrapment of the membrane-impermeant fluorophore 6CF. A similar protocol was used to label endosomes from the IMCD cells grown in primary culture and from the established epithelial cell lines MDCK, LLC-PK1 and A6. After 30 min of labeling, examination of each cell type by epifluorescence microscopy (excitation 480 nm, emission >515 nm) revealed multiple very small fluorescent bodies with the characteristic appearance of labeled endosomes (Lencer et al., 1990). There was no obvious effect of vasopressin or 8-Br-cAMP on

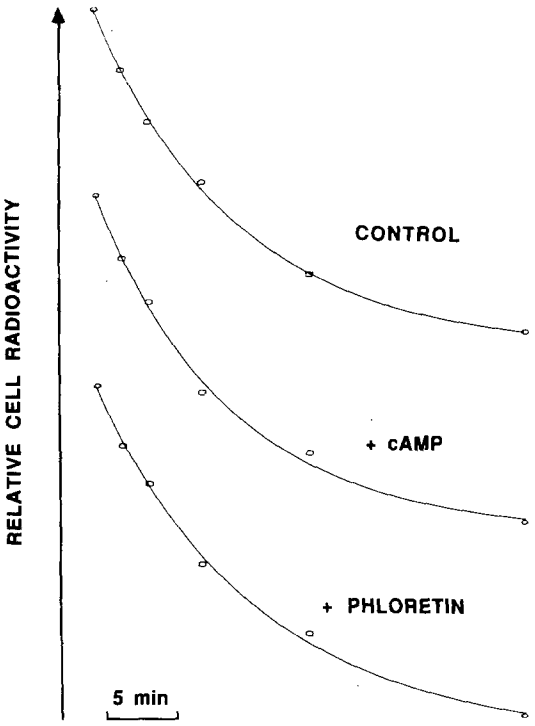

Fig. 5. ^{14}C-urea efflux in IMCD cells grown in primary culture. Efflux was measured at 37°C in monolayer cultured cells loaded with ^{14}C-urea as described in Materials and Methods. Phloretin (250 μM) was present only in the efflux buffer. 8-Br-cAMP (1 mM) was present 10 min before initiation of efflux and in the efflux buffer. Single-exponential fits are shown for data normalized to unity at zero time. Results of a series of paired studies are given in the text

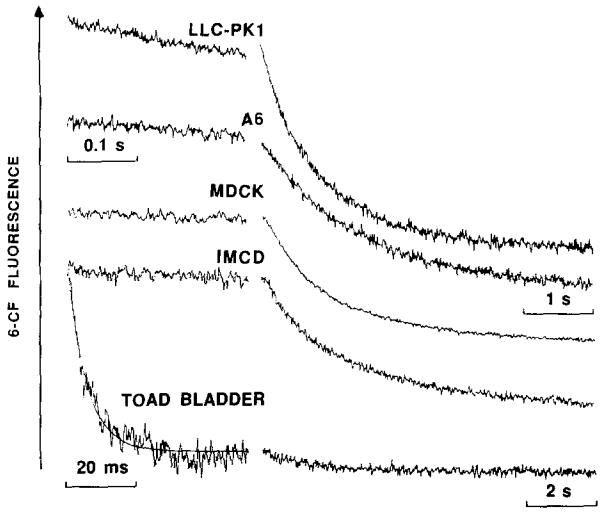

Fig. 6. Time course of water transport in endosomes from cultured kidney cells and toad urinary bladder. Endosomes were labeled with 6CF in intact cells, isolated, and subjected to a 60-mM inwardly directed sucrose gradient in a stopped-flow apparatus as described in Materials and Methods. 1 mU/ml vasopressin was present at the time of cell incubation with 6CF. Similar results were obtained in the absence of vasopressin and in the presence of 1 mM 8-Br-cAMP. Toad bladders were labeled with 6CF from the mucosal solution as described previously (Shi & Verkman, 1989). P_f values are given in the text

the number or cellular distribution of the labeled endosomes.

Figure 6 shows the time course of water permeability in endosomes from IMCD cells in the presence of vasopressin. Data at early times are shown on an expanded time scale to observe rapid water transport if present. The amplitude and time course of the slow fluorescence signal (P_f = 0.001) was not different than that measured in cells not treated with vasopressin, or cells treated with 1 mM 8-Br-cAMP (*not shown*). Water transport was also slow in the MDCK cells (P_f = 0.001 cm/sec), LLC-PK1 cells (P_f = 0.002 cm/sec) and A6 cells (P_f = 0.001 cm/sec). In contrast, parallel experiments in which apical vesicles of intact toad urinary bladder were loaded with 6CF in the presence of serosal vasopressin (Shi & Verkman, 1989) showed extremely fast water transport (P_f = 0.1 cm/sec). Therefore the endocytic retrieval of functional water channels, a characteristic of in vivo IMCD cells and toad urinary bladder, was absent in cultured IMCD cells and the other established renal epithelial cell lines.

Discussion

The purpose of this study was to characterize urea transport in freshly isolated and cultured IMCD cells. IMCD cells are the main cell type in the papillary collecting duct and are morphologically different from principal cells of more proximal collecting duct segments (Clapp et al., 1989). Urea transport in the freshly isolated cells was rapid, stimulated by cAMP agonists, and inhibited by phloretin, high affinity urea analogues and a protein kinase A inhibitor. These results provide strong evidence that a specialized urea carrier is expressed on the plasma membranes of freshly isolated IMCD cells in suspension and that urea transport is regulated by the cAMP pathway. The data do not address whether the mechanism of regulation involves intracellular trafficking of urea transporters or modulation of the activity of fixed plasma membrane urea transporters. Urea transport in the IMCD cells grown in primary culture was slow and not affected by phloretin and cAMP agonists. cAMP-dependent endocytosis of functional water channels was also absent. Attempts to maintain the expression of urea transport-

ers by modification of the stimulation protocol or the cell growth conditions were not successful.

It is interesting to compare urea permeability coefficients (P_{urea}) in lipid bilayers, red blood cells and the intact kidney. In the human red blood cell, where urea transport is mediated by a saturable carrier that is >95% inhibitable by phloretin (Macey, 1984; Dix et al., 1985), P_{urea} is 10^{-3} cm/sec. In pure lipid membranes in the form of a planar bilayer or spherical vesicle, P_{urea} is generally between 10^{-7} and 5×10^{-6} cm/sec, depending upon the membrane lipid composition and cholesterol content (Gallucci, Micelli & Lippe, 1971). In human platelets, a cell in which facilitated urea transport is absent, P_{urea} is 2×10^{-6} cm/sec (Meyer & Verkman, 1986). In intact IMCDs from rat kidney at 37°C, unstimulated P_{urea} was low in the outer third of the inner medulla (10^{-5} cm/sec) and high in the inner two-thirds (13×10^{-5} cm/sec) (Sands & Knepper, 1987). Urea transport was unaffected by 10^{-8} M vasopressin in the outer third of the medullary collecting duct, but increased to 70×10^{-5} cm/sec in the inner two-thirds (Sands, Nonoguchi & Knepper, 1987). Recent studies of P_{urea} in separate plasma membranes of rat IMCD gave a P_{urea} of 23×10^{-5} cm/sec (unstimulated) in the basolateral membrane which was several-fold greater than P_{urea} in the apical membrane (Star, 1990). If a membrane amplification factor of 5 is used, P_{urea} for the basolateral membrane is $\sim 5 \times 10^{-5}$ cm/sec.

It is difficult to estimate absolute P_{urea} for the suspended IMCD cells because most cells are in tubule fragments and clumps. Assuming a cell surface-to-volume ratio of 2200 cm^{-1} for a tubule fragment, a minimum value of P_{urea} corresponding to a 40-sec exponential time constant at 37°C is 10^{-5} cm/sec. The actual value of P_{urea}should be much greater than this because of the decreased surface-to-volume ratio in cell clumps and the presence of multiple serial barriers to urea diffusion. In addition, our cell preparation contained the complete papilla, of which ~50% was cellular material from the inner two-thirds of IMCD (Sands & Knepper, 1987).[1] Another important factor was that cell polarity is lost in suspended cells. Urea efflux through apical and basolateral membrane transporters was measured in parallel in membranes that may have become hybrid structures due to the loss of tight junctions. These factors account in part for the lower stimulation of J_{urea} by cAMP in the freshly isolated cells and for the lower inhibition of J_{urea} by 0.25 mM phloretin (>75% in intact IMCD; Chou & Knepper, 1989; 42% in the freshly isolated cells).

In the human red blood cell, very high urea permeability has been proposed to be important to prevent cell lysis during red cell passage through the hypertonic renal medullary interstitium and subsequent return to the isotonic cortical interstitium (Macey, 1984). Urea transport is saturable, inhibitable by phloretin and urea analogues, and inactivated by radiation (Mayrand & Levitt, 1983; Dix et al., 1985). The urea analogues chosen for the present study are extremely potent in the red cell; the K_I's for phenylurea and dimethylurea were 0.1 and 0.8 μM, respectively. A high concentration of 10 mM was chosen for these studies because of the lower potencies of other urea analogues in IMCD compared with red cells. Chou and Knepper (1989) reported that the K_I for thiourea was ~30 mM, higher than that of 12 mM in red cells.

Star et al. (1988) concluded that activation of urea transport in intact IMCD was mediated by cAMP and probably not by Ca. Our results support this conclusion. Vasopressin and 8-Br-cAMP stimulated urea transport in the freshly isolated cells, whereas the protein kinase A (cAMP-dependent protein kinase) blocker H-8 inhibited urea transport. There was no effect of protein kinase C activation by PMA, or phospholipase C activation by epidermal growth factor.

Although vasopressin stimulates both osmotic water and urea permeability in the inner two-thirds of the IMCD and the toad urinary bladder, there is increasing evidence that the pathways and regulatory mechanisms for water and urea transport are different. Knepper et al. (1989) reported that the urea reflection coefficient was near unity in the terminal IMCD, supporting the presence of separate physical pathways for water and urea transport. Shi et al. (1990) found that endosomes which carry the vasopressin-sensitive water channel in toad urinary bladder have low urea permeability and a urea reflection coefficient near unity. The biochemical identity of the kidney urea transporter and the mechanism by which urea transport is regulated are unknown at this time. Our studies suggest that mRNA obtained from freshly isolated IMCD cells should be suitable for expression cloning of the kidney urea transporter.

Development of cell culture models for vasopressin-sensitive water and urea transport is important for studies of transporter isolation and character-

[1] Separation of the rat papilla into outer and inner portions is difficult because of its small size. However, to investigate whether urea transport properties in freshly suspended cells were different within the papilla, papillas from five rats were cut into outer and inner portions of approximately equal mass. Urea transport measured under control conditions was not different in the two preparations, nor was there a significant difference in the percentage increase in J_{urea} in response to 1 mM 8-Br-cAMP (outer papilla, 32%; inner papilla, 34%).

ization, and of regulation at the transcriptional, translational and protein levels. The method for culture of IMCD cells used in our experiments is well established. The cells are >95% pure, have vasopressin-stimulated cAMP production (Teitelbaum & Berl, 1986), and EGF-stimulated phosphoinositol hydrolysis (Teitelbaum & Strasheim, 1989).

Unfortunately, the cultured IMCD cells lacked functional urea transporters, cAMP-dependent urea transport and cAMP-dependent endocytosis of water channels. It is not known whether urea carriers were absent because of culture conditions, preferential growth of proximal IMCD cells, or intrinsic cell properties. Expression of urea transport was not influenced by the time after cell plating or the composition of the growth medium. Growth of epithelial cells on a porous support might help to preserve the urea transport phenotype (Handler, Preston & Steele, 1984); however, in preliminary experiments the growth of a homogeneous population of IMCD cells was difficult using porous Nucleopore filters. Johnson et al. (1981) studied transepithelial water and urea transport in two cell lines from toad bladder epithelium. They found no stimulation of cAMP by vasopressin and no effect of 8-Br-cAMP on water transport. There was a twofold stimulation of urea transport by 1 mM 8-Br-cAMP, much less than that observed in intact bladder. Inhibitor effects were not examined. Alternative cell culture models must be evaluated which preserve vasopressin-sensitive water and urea permeability.

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