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Activation of Osmolyte Efflux from Cultured Renal Papillary Epithelial Cells

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Summary. The rabbit renal papillary epithelial cell line PAP-HT25 accumulates sorbitol and other organic osmolytes when cultured in hypertonic media. When returned to isotonic media, PAP-HT25 cells swell because of water influx and then shrink to their normal volume because of rapid osmolyte and water efflux (volume regulatory decrease, VRD). Sorbitol efflux from PAP-HT25 cells during VRD was reduced to 18% of control by incubation of the cells with 100 μM eicosatetraynoic acid (ETYA), indicating that an enzyme that metabolizes arachidonic acid (AA) is a key component of the efflux process. Sorbitol efflux was unaffected by incubation with cyclooxygenase and lipoxygenase inhibitors but was reduced to 9% by incubation with 100 μM ketoconazole and to 37% by incubation with 100 μM SKF-525A, indicating that the cytochrome P-450 limb of the AA cascade is involved in the efflux process. The efflux of other organic osmolytes betaine and myoinositol, but not glycerolphosphorylcholine, was also inhibited by incubation with ETYA and ketoconazole.

Key Words sorbitol · osmolytes · arachidonic acid · cytochrome P-450

Introduction

Renal medullary cells are able to withstand the large variations in extracellular osmolality that are part and parcel of the urine concentrating mechanism. This ability is believed to be largely due to the cells' capacity to accumulate osmotically active, yet chemically inert, organic solutes ("osmolytes") when the extracellular osmolality is increasing as well as their capacity to release these osmolytes when the extracellular osmolality is decreasing. By these means, deleterious variations in cell volume and the concentrations of important intracellular solutes are minimized [16].

Sorbitol, betaine, myoinositol and glycerolphosphorylcholine (GPC) are the osmolytes accumulated by renal medullary cells in vivo [1, 18] and in vitro [3, 26]. Sorbitol and GPC are produced by the cell [3, 27], whereas betaine and myoinositol are transported into the cell [28, 29]. Osmolyte accumulation and loss have been conveniently studied in vitro in the rabbit renal papillary epithelial cell line PAP-HT25 [2, 3, 17, 24, 41, 48]. In these cells, sorbitol, produced from glucose in a reaction catalyzed by aldose reductase, is the major osmolyte. In response to an initial increase in osmolality, aldose reductase and sorbitol levels rise slowly, reaching a peak after about four days. When the intracellular osmolality is then abruptly decreased, there is rapid efflux of sorbitol. Aldose reductase levels, however, decline slowly. Rapid regulation of intracellular osmolyte concentrations is, therefore, probably achieved by regulation of osmolyte efflux pathways [16].

Sorbitol efflux from PAP-HT25 cells has been well characterized [2, 17, 24, 41]. The efflux pathway is located in the apical membrane of the cell [17]. It is rapidly activated by a relative decrease in osmolality and deactivated by a relative increase in osmolality. It is not influenced by extracellular Na, K, or Ca substitution or by the membrane potential. A number of inhibitors of transport (ouabain, phloretin, phlorizin, probenecid, bumetanide, furosemide, amiloride, SITS, DIDS) or of the cytoskeleton (colchicine, cytochalasin B) failed to alter sorbitol release. The only inhibitor which was effective was quinidine at a relatively high concentration of 1 mM [41]. In addition, sorbitol efflux could not be activated in the absence of an osmolality change by ion substitutions or the addition of cyclic AMP analogues [41]. The efflux pathways for betaine, myoinositol and GPC have not been well characterized. Relative to sorbitol efflux, betaine efflux from PAP-HT25 cells is faster, whereas the effluxes of myoinositol and GPC are slower [24]. It is presumed that osmolyte efflux is mediated by one or more specific membrane-associated transport proteins.

Remarkably little is known about the ways in

which a cell perceives its volume and institutes processes that reverse swelling or shrinkage [21]. In particular, the activation of transporters responsible for osmolyte efflux from renal medullary cells is poorly understood. In the studies reported in this paper, we examined the mechanisms underlying the activation of osmolyte efflux from the cultured rabbit renal papillary epithelial cell line PAP-HT25. As sorbitol is the predominant osmolyte in this cell line and relatively easy to measure, we concentrated on the regulation of sorbitol efflux. We attempted to activate sorbitol efflux without changing the osmolality and to inhibit the sorbitol efflux associated with a sudden decrease in osmolality. We show that inhibitors of the production of cytochrome P-450 metabolites of arachidonic acid (AA) block the activation process.

Materials and Methods

Cell Culture

The PAP-HT25 line represents those cells from the GRB-PAP1 line that survived culture in hypertonic media [48]. The GRB-PAP1 line was derived from native rabbit renal papillary epithelium [48]. PAP-HT25 cells retain their phenotype when regrown in isotonic media [48].

PAP-HT25 cells were grown to confluence in 6-cm diameter plastic dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) at 37°C in 95% air, 5% CO_2 in isotonic (300 mOsm) media (9:1 Coon's modification of Ham's F-12: Liebowitz's L-15, containing 1% heat-inactivated calf serum, 1% heat-inactivated rabbit serum, transferrin and insulin (each 5 μg/ml), 5 pM triiodothyronine, 0.7 nM PGE_1, 5 nM selenium, and 50 nM hydrocortisone. Cells were then cultured in hypertonic medium (500 mOsm achieved with added NaCl) for at least four days, with medium changed daily. Under these conditions, the cells accumulated sorbitol, myoinositol and GPC. In some experiments, 0.5 mM betaine was added to the hypertonic medium, allowing the cells to accumulate betaine also. Cells from passages 85–114 were used.

Efflux Studies

Efflux experiments were performed at 37°C with the dishes being gently rotated on a rocker. Medium was first aspirated and the cells were washed once with 2 ml of warmed hypertonic buffer (500 mOsm) containing (in mM): 20 sodium HEPES (pH 7.4), 220 NaCl, 50 KCl, 1.8 NaH_2PO_4, 0.4 $MgSO_4$, 1.1 $CaCl_2$, and 10 D-glucose. Cells were then allowed to equilibrate in 2 ml of this buffer (with or without "inhibitors") for 30 min at 37°C, at which point an experiment was begun. The hypertonic buffer was aspirated and replaced with 2 ml of hypertonic buffer (with or without "activators") or isotonic buffer (300 mOsm, with or without "inhibitors"). Isotonic buffer was identical to hypertonic buffer except for the lower NaCl concentration. At the indicated times, the buffer was aspirated for measurement of sorbitol. Cells were dissolved in 4 ml of 0.5 M NaOH and assayed for protein. Osmolyte efflux, based on triplicate cell samples, is expressed as pmol/μg protein · min^{-1}.

In experiments in which cell osmolyte concentrations were measured, the buffer was aspirated at the appropriate time and cells were immediately washed with 2 ml of ice-cold hypertonic buffer to minimize subsequent osmolyte efflux.

Assays

Sorbitol was assayed with a kit (Boehringer-Mannheim, FRG, catalogue number 670,057). In this method, as sorbitol is converted to fructose by sorbitol dehydrogenase, iodonitrotetrazolium chloride is reduced to a formazan which is measured spectrophotometrically. GPC was assayed enzymatically [51] using GPC diesterase instead of phospholipase D. Betaine and inositol were measured by HPLC as previously described [50]. Protein was measured by the method of Bradford (8) using the BioRad reagent and bovine gamma globulin as standard (BioRad, Richmond, CA).

Chemicals

AA (5,8,11,14-eicosatetraenoic acid), ETYA (5,8,11,14-eicosatetraynoic acid), ETI (5,8,11-eicosatriynoic acid), EDYA (8,11-eicosadiynoic acid), DEDA (7,7-dimethyleicosadienoic acid), OOEP-choline (oleyloxyethyl phosphorylcholine, CDP-choline (cytidine 5'-diphosphocholine sodium salt), indomethacin, naproxen, NDGA (nordihydroguaiaretic acid), dipyridamole, baicalein (5,6,7-trihydroxyflavone), caffeic acid, curcumin, esculetin, gossypol, phenidone, acivicin, ketoconazole, SKF-525A, methoxasalen (8-methoxypsoralen), 5,6-EET (±5,6-epoxyeicosa-8Z,11Z,14Z,-trienoic acid), 8,9-EET (±8,9-epoxyeicosa-5Z,11Z,14Z,-trienoic acid), 11,12-EET (±11,12-epoxyeicosa-5Z,8Z,14Z,-trienoic acid), 14,15-EET (±14,15-epoxyeicosa-5Z,8Z,11Z,-trienoic acid), 5-HETE (±5-hydroxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid), 12(R)-HETE (12-R-hydroxyeicosa-5Z,8Z,10E,14Z,17Z-tetraenoic acid) and 15(S)-HETE (15-S-hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid) were purchased from BIOMOL Research Laboratories, Plymouth Meeting, PA; Quinacrine, 4-bromophenacylbromide, metyrapone, acetylsalicylic acid, melittin, A23187, dibutyryl cAMP, dibutyryl cGMP, phorbol 12-myristate 13-acetate, 5,6-DHA (5,6-dehydroarachidonic acid), GPC diesterase and leukotriene D_4 from Sigma Chemical, St. Louis, MO; [5,6,8,9,11,12,14,15-^{3}H-] AA (code TRK.757, batch 42) from Amersham, Arlington Heights, IL.

Stock solutions of hydrophobic compounds were made in dimethyl sulfoxide (Pierce, Rockford, IL) or ethanol (World Ethanol, Texas City, TX). The final concentration of these solvents in the experimental buffers were 0.1%. Controls contained dimethyl sulfoxide or ethanol at the same concentration.

Results

Sorbitol Efflux

When hypertonically adapted PAP-HT25 cells were equilibrated in hypertonic HEPES buffer (500 mOsm) and then transferred to the same hypertonic HEPES buffer there was little, if any, efflux of sorbitol (Fig. 1). When PAP-HT25 cells were transferred from hypertonic to isotonic HEPES buffer

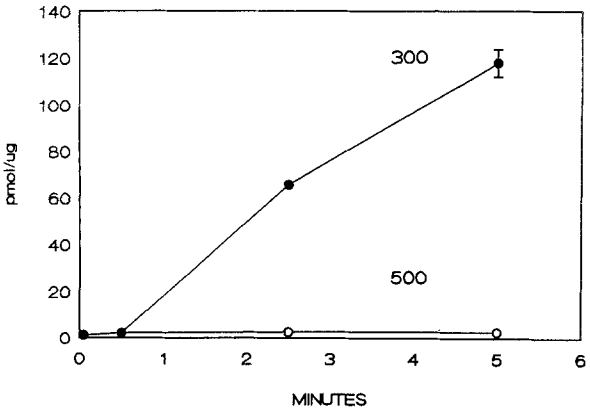

Fig. 1. Sorbitol efflux (pmol/μg protein) from hypertonically adapted PAP-HT25 cells. Cells were equilibrated in 500 mOsm HEPES buffer and then switched to 500 mOsm HEPES buffer (blank) or 300 mOsm HEPES buffer (control). Sorbitol efflux in the latter situation was linear for approximately 5 min. Results are mean ± SD from one experiment.

(300 mOsm), there was marked efflux of sorbitol (Fig. 1). Efflux commenced approximately 30 sec after the solution change, and the rate of efflux was essentially constant for the next 4.5 min. In most experiments, therefore, we measured sorbitol efflux in the 5-min period after the solution change. In agreement with previous observations [41], the removal of Ca^{2+} from the experimental buffers did not inhibit sorbitol efflux (*data not shown*). Sorbitol efflux was reduced slightly by 10 μM trifluoperazine, a putative calmodulin antagonist, while 100 μM caused greater inhibition (Table 1). Although the effect of high concentrations of trifluoperazine may be consistent with an involvement of calcium-calmodulin, all other experiments involving Ca^{2+} manipulations were negative. Sorbitol efflux in the absence of an osmolality change was not increased by 2 mM cAMP, 2 mM cGMP, 2 μM A23187 (a Ca^{2+} ionophore) or 0.3 μM phorbol 12-myristate 13-acetate (Fig. 2), leading us to conclude that cyclic nucleotides, Ca^{2+} and protein kinase C, were not involved in the activation of sorbitol efflux.

Inhibitors of AA Metabolism

Incubation of PAP-HT25 cells with 100 μM ETYA for 30 min before changing medium osmolality resulted in a sorbitol efflux equal to 18% of control (Table 1). The IC_{50} for ETYA was 51 μM. ETYA is a 20-carbon polyunsaturated fatty acid containing four acetylenic (triple) bonds at the positions C_{5-6}, C_{8-9}, C_{11-12} and C_{14-15}. AA, in contrast, contains four ethylenic (double) bonds in these positions (Fig. 3). Oxygenation of acetylenic bonds by cyclooxygenase, lipoxygenase and cytochrome P-450 results in the production of compounds that irreversibly inactivate the oxygenase [11, 12, 42]. The inhibitory effect of ETYA indicated, therefore, that an enzyme that metabolizes AA at its ethylenic bonds is a key component of the mechanism underlying sorbitol efflux. Although it is hypothesized that the availability of AA is rate limiting for the production of metabolites [22], incubation of the cells with 100 μM AA failed to influence sorbitol efflux.

We then tested the effects of other acetylenic derivatives of AA (Fig. 3 and Table 1). Incubation witH ETI, a triacetylenic derivative lacking the C_{14-15} triple bond, reduced sorbitol efflux to 15% of the control value. EDYA, a diacetylenic derivative

Table 1. Effects of various inhibitors on sorbitol efflux from hypertonically adapted PAP-HT25 cells[a]

Inhibitor	μM	% Control efflux	Experiments
1. Trifluoperazine	10	70	1
	100	26	2
2. Arachidonic acid	100	105 ± 6	3
ETYA	100	18 ± 9	9
ETI	100	15 ± 13	3
EDYA	100	69 ± 3	3
5,6-DHA	100	29	2
3. Indomethacin	500	77	1
	100	90	2
Naproxen	100	101	1
Acetylsalicylic acid	100	103	1
4. NDGA	315	48	1
	100	62 ± 26	3
	50	104	1
Dipyridamole	100	98	1
Baicalein	100	82	1
Caffeic acid	100	126	2
Curcumin	100	67	2
Esculetin	100	91	1
Phenidone	500	98	1
Gossypol	1	102	1
Acivicin	2000	101	1
5. Ketoconazole	100	9 ± 7	3
SKF-525A	315	19	1
	100	37 ± 8	6
Methoxasalen	100	95 ± 14	3
Metyrapone	100	96	1
6. Quinacrine	500	27	2
	100	71	2
4-Bromophenacylbromide	500	9	2
	100	70	2
OOEP-choline	100	105	1
DEDA	100	100	1
CDP-choline	200	104	1

[a] Cells were equilibrated in 500 mOsm HEPES buffer and then transferred to 500 mOsm HEPES buffer (blank) or to 300 mOsm HEPES buffer with or without (control) inhibitors. The cells were preincubated with each compound for 30 min. Results are mean ± SD of triplicate observations for each experiment.

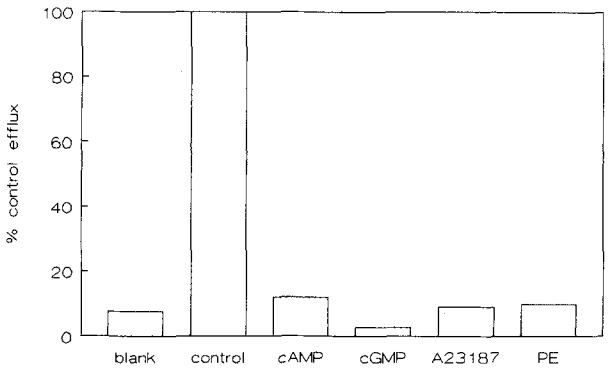

Fig. 2. The efflux of sorbitol, as a percentage of the control value, from cells equilibrated in a 500-mOsm HEPES buffer and then switched to the same buffer (blank), a 300 mOsm HEPES buffer (control), or a 500 mOsm HEPES buffer containing 2 mM cAMP, 2 mM cGMP, 2 μM A23187 in the presence of 1.1 mM Ca^{2+}, or 0.3 μM phorbol 12 myristate 13 acetate (PE). All bars represent the mean of a single experiment with triplicate observations.

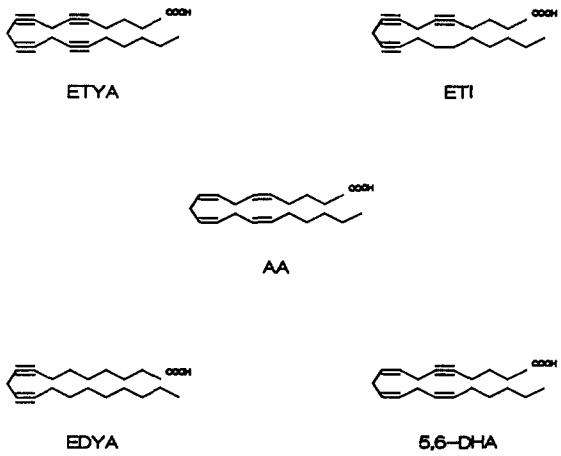

Fig. 3. The structures of the acetylenic derivatives of AA used as inhibitors in this study.

lacking the C_{5-6} and C_{14-15} triple bonds, slightly reduced sorbitol efflux to 69% of the control value. Incubation with 5,6-DHA produced significant inhibition, with sorbitol efflux only 29% of control.

AA, usually liberated from membrane phospholipids by the action of phospholipase A_2 [22, 30, 49], is metabolized in reactions catalyzed by cyclooxygenase, lipoxygenase and cytochrome P-450 to biologically active eicosanoids or reincorporated into membrane phospholipids in reactions catalyzed by arachidonyl CoA synthetase and an acyl transferase [22, 30]. ETYA has been reported to inhibit all but the acyl transferase activities [7, 9, 13, 37, 46, 47]. We therefore used specific inhibitors to determine which of these steps was involved in the activation of sorbitol efflux (Table 1).

Cyclooxygenase Inhibitors

Incubation with 100 μM indomethacin, naproxen or acetylsalicyclic acid, known cyclooxygenase inhibitors, did not inhibit sorbitol efflux (Table 1). Curcumin and phenidone, agents with inhibitory effects on both cyclooxygenase and lipoxygenase, also had no effect. These results indicated that prostaglandins, prostacyclins and thromboxanes were not involved in the activation of sorbitol efflux.

Lipoxygenase Inhibitors

NDGA, a 5-lipoxygenase inhibitor, produced moderate inhibition of sorbitol efflux (Table 1). Efflux, however, was not inhibited by other 5-lipoxygenase inhibitors (dipyridamole, curcumin, esculetin, phenidone and caffeic acid), by 12-lipoxygenase inhibitors (baicalein, gossypol and esculetin) or by 15-lipoxygenase inhibitors (gossypol). As leukotrienes are the major biologically active products of the lipoxygenation of AA [30], we sought further evidence that these compounds were not involved in the activation of sorbitol efflux. LTA_4 is conjugated with glutathione to form LTC_4. LTC_4 is converted to LTD_4 by γ-glutamyl transferase. The γ-glutamyl transferase inhibitor acivicin did not influence sorbitol efflux, indicating no role for LTD_4, LTE_4 or LTF_4.

Cytochrome P-450 Inhibitors

As shown in Table 1, sorbitol efflux was markedly inhibited by the cytochrome P-450 inhibitors ketoconazole and SKF-525A [9, 43]. 100 μM ketoconazole reduced sorbitol efflux to 9% of control with an IC_{50} of 45 μM. Methoxasalen and metyrapone, inhibitors of other cytochrome P-450 enzymes, had no effect. These results suggested that a ketoconazole-sensitive cytochrome P-450 metabolizes AA in PAP-HT25 cells during VRD. Furthermore, the results suggested that the metabolite(s) activated the sorbitol transporter.

Phospholipase A_2 Inhibitors

Incubation of PAP-HT25 cells with a 100 μM concentration of the phospholipase A_2 inhibitors quinacrine or 4-bromophenacylbromide caused a 30% inhibition of sorbitol efflux. Relatively high concentrations (500 μM) caused substantial inhibition (Table 1). Incubation with 100 μM OOEP-choline,

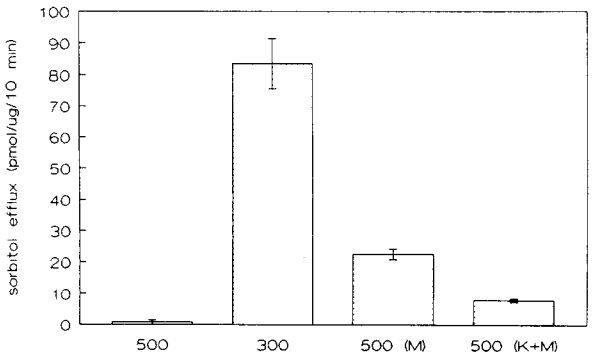

Fig. 4. The effect of 1 μM melittin on sorbitol efflux (pmol/μg protein) from hypertonically adapted PAP-HT25 cells. Cells were equilibrated in 500 mOsm HEPES buffer and then transferred to 500 mOsm HEPES buffer (*500*), 300 mOsm HEPES buffer (*300*), 500 mOsm HEPES buffer containing 1 μM melittin (*500(M)*] or preincubated in 500 mOsm HEPES buffer containing 100 μM ketoconazole and then transferred to 500 mOsm HEPES buffer containing 1 μM melittin (*500(K + M)*]. Results are mean ± SD from one experiment (triplicate observations).

DEDA or 200 μM CDP-choline, other reputed phospholipase A_2 inhibitors, had little effect. Although these results are consistent with the conclusion that availability of AA may be limited by inhibition of phospholipase A_2, the high concentrations required cast some doubt upon the validity of this conclusion.

Stimulation of Sorbitol Efflux by Phospholipase A_2 Activation

Hypertonically adapted PAP-HT25 cells were transferred to hypertonic buffer containing 1 μM melittin, a phospholipase A_2 activator, which produced a significant efflux of sorbitol (Fig. 4). In addition, melittin-stimulated release of sorbitol was partially inhibited by prior incubation of the cells with 100 μM ketoconazole. These results indicated that the effect of melittin was not solely a consequence of a nonspecific increase in membrane permeability. At higher concentrations of melittin, we observed a greater stimulation of sorbitol efflux and, in some experiments, cell detachment. Under these conditions, efflux was less sensitive to prior incubation with ketoconazole, suggesting that higher concentrations of melittin caused a nonspecific increase in sorbitol permeability. Although both activation and inhibition of phospholipase A_2 influenced the magnitude of the sorbitol permeability, the high concentrations of inhibitors required and the fact that the effect of melittin was always considerably less than that of a reduction in osmolality suggested that phospholipase A_2 activation may play only a minor role during RVD.

Table 2. PAP-HT25 cell and buffer osmolyte levels at 5 and 10 min following a sudden reduction of osmolality[a]

	Inositol	GPC	Sorbitol	Betaine
Cell (5 min)				
Blank	62 ± 3	25 ± 1	327 ± 12	62 ± 2
Control	58 ± 4	19 ± 2	267 ± 9	48 ± 1
ETYA	63 ± 3	15 ± 4	314 ± 25	56 ± 4
Indomethacin	60 ± 4	21 ± 2	280 ± 15	51 ± 6
NDGA	58 ± 0.4	19 ± 3	300 ± 10	54 ± 4
Ketoconazole	61 ± 2	18 ± 1	329 ± 6	61 ± 3
Buffer (5 min)				
Blank	0	—	1 ± 0.2	4 ± 4
Control	4 ± 0.3	—	37 ± 4	18 ± 3
ETYA	0	—	2 ± 0.2	2 ± 0.1
Indomethacin	5 ± 1	—	42 ± 6	20 ± 1
NDGA	1 ± 1	—	14 ± 3	7 ± 1
Ketoconazole	1 ± 0.4	—	3 ± 1	2 ± 1
Cell (10 min)				
Blank	79 ± 5	25 ± 1	320 ± 14	63 ± 3
Control	56 ± 1	15 ± 5	182 ± 17	13 ± 1
ETYA	75 ± 7	8 ± 8	293 ± 13	48 ± 8
Indomethacin	53 ± 3	18 ± 1	173 ± 5	15 ± 1
NDGA	71 ± 8	17 ± 5	246 ± 18	47 ± 12
Ketoconazole	77 ± 1	3 ± 2	310 ± 18	68 ± 3
Buffer (10 min)				
Blank	1 ± 0.1	—	2 ± 0.1	1 ± 0.2
Control	19 ± 0.4	—	144 ± 14	48 ± 1
ETYA	5 ± 1	—	32 ± 9	13 ± 3
Indomethacin	21 ± 2	—	147 ± 6	47 ± 2
NDGA	8 ± 0.2	—	60 ± 11	28 ± 2
Ketoconazole	3 ± 1	—	6 ± 1	3 ± 1

[a] The conditions are the same as in Table 1. Results are mean ± SD from two experiments (5 and 10 min) each involving triplicate observations.

Effects on Other Osmolytes

Although sorbitol is the major osmolyte in hypertonically adapted PAP-HT25 cells, there is also significant accumulation of myoinositol and GPC, and, if it is present in the medium, betaine [24]. To test whether or not the efflux of these other osmolytes was inhibited by ETYA and ketoconazole, the standard experiments were performed and cell and buffer osmolyte concentrations were measured using HPLC (Table 2). Significant inhibition of betaine and myoinositol efflux was seen 5 and 10 min after incubation with ETYA and ketoconazole. Moderate inhibition was seen after incubation with NDGA, whereas no inhibition was seen with indomethacin. No inhibition of GPC efflux was seen with these agents. Somewhat surprisingly, GPC disappeared from cells more rapidly in the presence of ETYA and ketoconazole. These results were especially pronounced at 10 min. We were unable to measure GPC in the buffer because of comigration

of GPC and dimethyl sulfoxide on chromatography. Enzymatic analysis failed to demonstrate GPC in the buffer; choline, however, was detected in both the buffer and within the cells incubated with ETYA and ketoconazole.

Attempts to Identify the AA Metabolites Involved

In preliminary experiments, the AA in the cells was labeled by adding 3.5 μM [^{3}H]-AA to the hypertonic medium for four days, and a methanol extract was dried under N_2 and analyzed by HPLC. A small peak was noted in a region usually occupied by the EETs (retention time = 25–28 min), representing approximately 0.5–1.5% of the label. No evidence of prostaglandins, leukotrienes, HETE or OH-AA production was seen. EETs are only produced in cytochrome P-450 catalyzed reactions. Based on these preliminary observations, we attempted to activate sorbitol efflux from hypertonically adapted PAP-HT25 without changing osmolality by adding aracidonic acid (100 μM), or P-450 metabolites (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5-HETE, 12-HETE, 15-HETE, all at a concentration of 1 μM), or leukotriene (LTD_4, 0.06 μM) to the culture medium. However, none of these compounds increased sorbitol efflux above background levels.

Discussion

To define the mechanisms underlying the activation of these osmolyte efflux pathways, we attempted to stimulate sorbitol efflux from hypertonically adapted PAP-HT25 cells without changing the osmolality and to inhibit the sorbitol efflux that occurs when the osmolality is reduced. Initial studies indicated that cAMP, cGMP, Ca^{2+} and protein kinase C had no role in the activation process. Previous work [41] also showed that cAMP and Ca^{2+} were not involved. A recent study with isolated rat inner medullary collecting duct cells [6] found, in contrast, a relationship between Ca^{2+} and sorbitol efflux. Efflux was stimulated twofold by 10 μM A23187 and inhibited approximately 60% by the addition of 20 μM trifluoperazine or removal of Ca^{2+} from the medium [6]. We saw modest inhibition (30%) of sorbitol efflux by 10 μM trifluoperazine, but question whether this effect is solely a consequence of its action as a calmodulin inhibitor. Trifluoperazine has been reported to act synergistically with ketoconazole in some in vitro systems [5], raising the possibility that its inhibitory effect on sorbitol efflux may be related to cytochrome P-450 inhibition rather than calmodulin inhibition. Since the sorbitol experiments were performed in different tissues and species, it is not surprising that differences in the regulatory mechanisms for sorbitol efflux exist.

The AA cascade is known to generate a large number of biologically active lipids in many cells [30]. ETYA is a tetraacetylenic derivative of AA. When the acetylenic bonds in ETYA are metabolized by an oxygenase, the enzyme is irreversibly inactivated. Inactivation is believed to result from the production of allenic hydroperoxides and OH· which oxidize the catalytic site [11, 12] or from the production of conjugated allenes which alkylate the catalytic site [42]. We found that incubation of PAP-HT25 cells with relatively low concentrations of ETYA resulted in inhibition of sorbitol efflux. The ETYA IC_{50} is probably an overestimation of the true value, as lipids are known to adsorb to the walls of test tubes and culture dishes [31]. This result indicated that an enzyme system that metabolizes AA (cyclooxygenase, lipoxygenase or cytochrome P-450) was a crucial component of the sorbitol efflux process. Furthermore, it indicated that a metabolite of AA probably activated the sorbitol transporter.

We then tested the effects of other acetylenic derivatives of AA. ETI, a triacetylenic derivative was as effective as ETYA. ETI has been used as a cyclooxygenase and lipoxygenase inhibitor [19, 35]. EDYA, a diacetylenic derivative, had only a slight inhibitory effect, whereas 5,6-DHA, a monoacetylenic derivative, had a marked inhibitory effect. EDYA has been used as a cyclooxygenase inhibitor [23] and 5,6-DHA as a 5-lipoxygenase inhibitor [11]. Although the effects of ETI, EDYA, and 5,6-DHA on cytochrome P-450 have not been studied, the inhibitory effect of 5,6-DHA suggests that the C_{5-6} bond of AA is oxygenated during VRD by PAP-HT25 cells.

Cyclooxygenase inhibitors had no effect on sorbitol efflux excluding a role for the prostaglandins, prostacyclins and thromboxanes. Lipoxygenase inhibitors, in general, had little effect, although NDGA had a moderate inhibitory effect, consistent with a role for 5-lipoxygenase.

Inhibitors of some cytochrome P-450s [21, 27] had either no effect (metyrapone, methoxasalen) or were markedly inhibitory (ketoconazole, SKF-525A). As cytochrome P-450 inhibitors are isozyme specific, these results indicate that a ketoconazole-sensitive cytochrome P-450 metabolizes AA to EETs, HETEs and OH-AAs in PAP-HT25 cells during VRD. As OH-AA production is not inhibited by ketoconazole [9], it is likely that an EET or HETE activates the sorbitol transporter. It is noteworthy that NDGA has inhibitory effects on cyto-

chrome P-450 [9] and that ketoconazole has inhibitory effects on 5-lipoxygenase [4], indicating that conclusions based solely on the effects of these inhibitors must be tentative.

AA release from membrane phospholipids is believed to be the rate-limiting step in eicosanoid production [22]. As AA is usually in the *sn*-2 position of membrane phospholipids, its release depends on the action of phospholipase A_2 [30, 49]. High concentration of inhibitors of this enzyme blocked sorbitol efflux, perhaps reflecting different sensitivities of the extracellular and intracellular forms of the enzyme [49]. If phospholipase A_2 inhibitors reduced sorbitol efflux when extracellular osmolality was decreased, phospholipase A_2 activators should stimulate sorbitol efflux in the absence of an osmolality change. 1 μM melittin modestly stimulated sorbitol efflux without a reduction in osmolality. This effect was partially inhibited by ketoconazole, indicating that we were not observing the sole result of a nonspecific increase in membrane permeability. However, neither the inhibitor nor activator results convincingly demonstrated a role for phospholipase A_2 as the key regulatory step in sorbitol release.

Our preliminary attempts to identify the specific AA metabolites by HPLC showed that PAP-HT25 cells metabolized AA to EETs but not to prostaglandins, leukotrienes, HETES or OH-AA's. As EETs are produced only in reactions catalyzed by cytochrome P-450 we had obtained initial confirmation of our hypothesis. We were unable to identify the specific EETs produced by PAP-HT25 cells, as 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET were not separable with the HPLC used. We were also unable to determine major differences between cells exposed to a reduction in osmolality and those not exposed. We anticipate that differences will emerge when the specific metabolites are identified and quantified.

Although long recognized that the cyclooxygenase and lipoxygenase products of AA are biologically active [30], it has only recently been recognized that cytochrome P-450 metabolites of AA are also active [15]. Similarly, although it has been known for many years that the cells in the renal medulla produce prostaglandins [30], only recently has it been recognized that these cells produce cytochrome P-450 metabolites of AA [10, 25, 32–34]. The major products in both rabbit and rat are OH-AAs, whereas the minor products are EETs. Although a variety of actions have been ascribed to these metabolites [14, 15, 20, 39, 40, 45], some doubt remains as to their true biological function [15].

The apparent failure of AA, EETs or HETEs to stimulate sorbitol efflux in the absence of an osmolality change would seem to undermine our conclusions concerning the importance of these compounds in the control of sorbitol efflux. However, these metabolites are hydrophobic and unstable in aqueous solutions and we may not have delivered the compounds in the correct concentrations or forms to the correct site in the cell. We have not, to date, been able to ascertain whether the addition of metabolites (in DMSO) to the aqueous medium above the cultured cells actually results in an increase in the intracellular concentrations of these substances. Alternatively, the response of hypertonically adapted PAP-HT25 cells to a sudden reduction in osmolality may be more complicated than conceived. For example, a number intracellular proteins have important inhibitory and stimulatory roles in the activation of phospholipase A_2 [49]. These proteins may have other functions in the AA cascade which we cannot emulate by the simple addition of EETs and HETEs. It is also known that 5-lipoxygenase activation can be blocked by preventing the enzyme's translocation from the cytosol to the plasma membrane [36]. Although cytochrome P-450 activities have been found in the plasma membrane [38, 39, 44], they are predominantly microsomal enzymes. Similar translocation events may also be important in PAP-HT25 cells during VRD.

Although sorbitol is the major osmolyte in hypertonically adapted PAP-HT25 cells and in rabbit papillae, it is not the major osmolyte in other renal cell lines or in other species [16, 21]. It was important, therefore, to determine whether or not the efflux of betaine, myoinositol or GPC was inhibited by the agents that inhibited sorbitol efflux. Betaine and myoinositol efflux were similarly inhibited by ETYA and ketoconazole, indicating the existence of a common activation mechanism presumably involving cytochrome P-450 metabolites of AA. In contrast, the disappearance of GPC, which was most likely a result of intracellular degradation to choline and other products, was accelerated by ETYA and ketoconazole. These results appear inconsistent with earlier work [24] which demonstrated efflux of GPC from PAP-HT25 cells after a reduction of osmolality. GPC accumulates in PAP-HT25 cells cultured in hypertonic media because of a relative inhibition of GPC diesterase under these conditions [51]. In the experiments reported here, relatively small amounts of GPC were accumulated by the cells making the results difficult to interpret. It is possible to increase the amount of GPC accumulated by PAP-HT25 cells [24], and the effects of ETYA and ketoconazole should be investigated under these conditions.

In conclusion, we have provided strong evidence that cytochrome P-450 metabolites are crucially involved in the release of organic osmolytes by renal papillary epithelial cells in culture. It remains to identify the specific EET involved in the activation of the PAP-HT25 cell sorbitol transporter and to show that these events occur in vivo.

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