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Metabolism of the 'organic osmolyte' glycerophosphorylcholine in isolated rat inner medullary collecting duct cells. II. Regulation by extracellular osmolality

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In isolated inner medullary collecting duct (IMCD) cells requirements for the organic osmolyte glycerophosphorylcholine (GPC) vary with extracellular osmolality. To investigate mechanisms of osmotic adaptation GPC metabolism was studied under different osmotic conditions. In contrast to the GPC precursors choline and phosphatidylcholine (PC) cellular GPC was proportional to the osmolality. Hypotonic decrease in cellular GPC was mediated by fast initial release significantly exceeding the low hypertonic release. In long-term studies the total amount of GPC decreased significantly under hypotonic conditions but remained constant under hypertonic conditions resulting in a significant difference after 15 h. To investigate osmotic influences on GPC synthesis and GPC degradation studies with [*methyl*-^{3}H]choline were performed. Pulse-chase experiments displayed no significant osmotic differences in PC synthesis or in PC degradation to GPC indicated by a similar specific activity of PC. This suggested that phospholipase A_2 (PC degradation) was osmotically insensitive. A small and distinct metabolic PC pool may be responsible for high radioactive labeling of newly synthesized GPC which displayed a significantly higher specific activity under hypotonic conditions accompanied by a decrease in GPC amount. Therefore, a higher activity of glycerophosphorylcholine : choline phosphodiesterase (GPC : choline phosphodiesterase) (GPC degradation) under hypotonic conditions is proposed. Similar conclusions can be drawn from using phosphatidyl[*methyl*-^{3}H]choline. As further evidence for osmotic regulation of GPC : choline phosphodiesterase the specific activity of choline displayed a significant hypotonic increase with chase time which may be equivalent to increased GPC degradation. Therefore, the in vitro experiments suggest that cellular GPC is regulated by an osmosensitive GPC : choline phosphodiesterase. Such a regulation also seems to be present during long-term in vivo experiments. No evidence was found for a genetic adaptation of GPC : choline phosphodiesterase in vivo.

Introduction

Renal inner medullary collecting duct cells are responsible for final alterations in the urine composition before it is released into the renal pelvis and the bladder [1]. Since the hydration status of organisms fluctuates depending on water intake and loss, urine osmolality and as a result the cellular environment of certain kidney cells can differ substantially [2–5]. For that reason these cells possess mechanisms to protect their integrity against osmotic challenges thus maintaining normal cellular functions. An essential strategy of IMCD cells is the use of organic osmolytes like GPC, sorbitol and others (e.g., Refs. 5 and 6) which do not perturb cell metabolism [7]. Under hypertonic conditions in vivo organic osmolytes can be rapidly synthesized (mainly sorbitol) or possibly taken up from interstitial compartments. Under hypotonic conditions synthesis and/or uptake are replaced by release of osmolytes into interstitial compartments. In vitro hypotonic surroundings usually cause massive release of GPC as well as other osmolytes whereas in hypertonic surroundings release is quite low (e.g., Refs. 8–10). Additionally, IMCD cells are able to perform a slow GPC net synthesis which is increased in case of requirements. The ability of isolated IMCD cells to metabolize GPC has been proven lately [11–13]. For *synthesis* the 'Kennedy pathway' from choline to PC [5,13–17] is used followed by a successive hydrolysis of the fatty acid residues of PC resulting in production of

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GPC. *Degradation* is catalyzed by GPC: choline phosphodiesterase forming choline and phosphoglycerol [13,16,17]. However, net synthesis of GPC under normal conditions is slow [18,19].

More than three decades ago, Ullrich [20] proposed that inhibition of the GPC degrading enzyme GPC: choline phosphodiesterase by high extracellular NaCl and urea causes an increase in intracellular GPC. Detailed studies on Madin Darby canine kidney (MDCK) cells by Zablocki et al. [21] failed to detect significant osmotic influence on the GPC synthesizing enzyme phospholipase A_2; they provided, however, additional evidence for GPC : choline phosphodiesterase being regulated by NaCl and urea thus controlling cellular GPC. In this paper we investigated the role of phospholipase A_2 and GPC: choline phosphodiesterase in the osmotic adaptation of isolated rat renal inner medullary collecting duct cells.

The studies presented in this article are part of the Ph.D. thesis of H.G. Bauernschmitt and have been published in abstract form [11,22].

Materials and Methods

Isolation of inner medullary collecting duct cells. Male Wistar rats (200–300 g) were killed by cervical dislocation, the white inner medulla of the kidneys was rapidly removed and placed into ice-cold 0.6 osmol/l Hepes Ringer's solution of the following composition (in mM): 268 NaCl, 16 Hepes, 16 Na Hepes, 5 glucose, 3.2 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$, 1.8 KH_2PO_4 (pH 7.4) [23]. After mincing the tissue, pieces were incubated in the same buffer with addition of 0.2% collagenase, 0.2% hyaluronidase and 0.001% DNase at 37 °C gasing with room air for 75 min. IMCD cells were isolated by repeated low-speed differential centrifugation as described previously [24]. The final cell preparation contained > 95% collecting duct cells. To investigate osmotic influences on GPC metabolism, buffer osmolality was lowered to 0.3 osmol/l by reducing NaCl to a final concentration of 118 mM during the experiments. Alternatively, buffer osmolality was increased to 0.9 osmol/l by adding NaCl to a final concentration of 418 mM. All incubations were at 37 °C for time periods given in the Results section. Viability of the cells was routinely checked by determination of the activity of lactate dehydrogenase (LDH, intracellular marker enzyme) in cell supernatants [23]. For up to 3 h, viability was between 90% (0.3 osmol/l) and 95% (0.9 osmol/l) followed by a linear decrease to 70% (0.3 osmol/l) or 85% (0.9 osmol/l) after 15 h.

Investigations with [methyl-^{3}H]choline and phosphatidyl[methyl-^{3}H]choline. In pulse experiments with [*methyl*-^{3}H]choline, 0.37 MBq/sample was used whereas pulse-chase experiments with [*methyl*-^{3}H]choline were performed with 1.48 MBq/sample. The choline concentration amounted to 165 nM (pulse) and 650 nM (pulse chase). In pulse and pulse-chase experiments with phosphatidyl[*methyl*-^{3}H]choline, 0.37 MBq/sample was added to the incubation medium containing IMCD cells as a 50% ethanolic solution (final ethanol concentration < 1%). The PC concentration amounted to 200 nM. In pulse experiments the radioactive precursor was presented to the cells for the indicated time periods (up to 15 h). The pulse medium always had an osmolality of 0.9 osmol/l to avoid cellular loss of radioactive metabolites, e.g., GPC. In pulse-chase experiments the radioactive precursor was removed after 30 min by sedimenting the cells for 3 min at $28 \times g$ at 4 °C. Cells were subsequently washed once with 1 ml Hepes buffer (0.9 osmol/l) using the same centrifugation procedure. This was followed by resuspension of cells in the appropriate chase medium. In [*methyl*-^{3}H]choline experiments the concentration of unlabeled choline in the chase amounted to 200 μM. In phosphatidyl[*methyl*-^{3}H]choline experiments no unlabeled PC was added in the chase. All incubations took place in a volume of 800 μl containing approximately 0.4 mg of protein. Finally, cells were separated from the incubation medium by centrifgation for 10 min at $1500 \times g$ at 4°C. This was done to analyse labeled cellular and extracellular metabolites separately. Samples were analysed either immediately or after storage at −20°C.

Lipid extraction. Lipids were extracted from cells and cell supernatants using methanol/trichloromethane/water (1:1:1, v/v) according to the method of Bligh and Dyer [25]. After separation of the upper layer mainly containing methanol and water (with non-lipids) from the lower layer mainly containing trichloromethane (with lipids) the solvents were evaporated in a vacuum centrifuge. Pellets containing lipids were resuspended in a small volume of trichloromethane/methanol (1:1, v/v) those containing non-lipids were resuspended in water.

Thin-layer chromatography. For water soluble metabolites a solvent system consisting of methanol, 0.5% NaCl and ammonia (100:100:4, v/v) was used (modified after Refs. 26 and 27). Lipid metabolites were separated in a solvent system consisting of trichloromethane, methanol, acetic acid and water (100:39:3:6, v/v) (modified after Ref. 28). Silica gel 60-plates (Merck, Darmstadt, Germany) were used for chromatography. Development of chromatograms took between 1.5 and 2 h before being dried for 30 min in a cold air stream. For identification of labeled metabolites of choline or PC, pure standards were run on the same chromatograms and stained with iodine vapor comparing the R_f values with those obtained in the experiments.

Autoradiography. The dry chromatograms were placed in the measuring chamber of a digital autora-

diograph (Berthold, Bergisch-Gladbach, Germany). After 2 h at a voltage of 1800 V in a saturated methylal atmosphere in argon/methane (90:10, v/v) at 3 °C specific signals emitted from the radioactive spots differed sufficiently from unspecific background. The radioactivity of each metabolite was determined as cpm. To transform cpm into dpm values, radioactive standards were used and the originally applied radioactivity was related to the measured radioactivity using these factors to correct for quench. Recovery of the radioactivity initially applied onto the chromatographic plate usually amounted to 94 ± 4%.

Determination of choline, phosphatidylcholine and glycerophosphorylcholine. GPC was determined by the method of Wirthensohn and Guder [29]. The procedure is based on acid hydrolysis of GPC to choline. In the presence of choline kinase and ATP choline is phosphorylated to phosphorylcholine. Successive enzymatic steps finally lead to an equimolar decrease of NADH which can be measured photometrically. Choline and PC were measured in the same tissue samples after lipid extraction. Choline was determined by the procedure of Wirthensohn and Guder in nonhydrolysed samples [29]. The principle of measurement for PC quantification is the hydrolysis to phosphorylcholine by phospholipase C followed by dephosphorylation of phosphorylcholine to choline by alkaline phosphatase. After inactivation of these enzymes the method is identical with the choline determination. A commercially available test kit was employed for PC determination (Boehringer, Mannheim, Germany).

Lactate dehydrogenase (LDH) and protein determination. LDH activity in cell supernatants was measured with a commercially available test kit at 37°C. Total cellular LDH activity was calculated after cell lysis of a sample aliquot at the start of incubation and amounted to 3148 ± 255 U/g protein. Protein was measured in triplicate according to Lowry et al. [30]. Sample and standard proteins were precipitated with 10% ice-cold trichloroacetic acid. Bovine serum albumin at concentrations between 0.2 and 0.6 mg/ml was used as standard.

Statistical analysis. Mean values with their corresponding standard error of mean (S.E.) of at least four independent experiments performed in duplicate are given throughout this paper. For statistical analysis paired or unpaired Student's *t*-test was employed, considering a difference at $P < 0.05$ as significant.

Materials. [*methyl*-^{3}H]Choline (2.81 TBq/mmol) was purchased from Amersham (Germany). Phosphatidyl[*methyl*-^{3}H]choline (L-α-dipalmitoyl) (1.11 TBq/mmol) was supplied from NEN Dupont (Germany). Radioactive compounds were checked for purity by thin-layer chromatography. They migrated as single spots indicating the lack of radioactive contaminants. All chemicals were of highest purity commercially available and were purchased from the German divisions of Sigma, Boehringer Mannheim, Fluka and Riedel de Haën unless otherwise stated.

Results

Effect of extracellular osmolality on total amount and cellular content of choline, phosphatidylcholine and glycerophosphorylcholine

At the start of the experiment total choline amounted to 56 ± 6.1 μmol/g protein. In the hypotonic medium choline content remained constant up to 3 h and increased significantly to 82 ± 8.8 μmol/g protein after 9 h ($P < 0.05$), and to 139 ± 18.2 μmol/g protein after 15 h ($P < 0.005$). In the hypertonic medium the increase in choline content was significantly higher. After 3 h the amount of choline increased significantly to 97 ± 10.1 μmol/g protein ($P < 0.05$), after 9 h to 124 ± 13.8 μmol/g protein ($P < 0.05$), and after 15 h to 190 ± 22.6 μmol/g protein ($P < 0.005$). During the first 20 min about 60% of choline was released into the supernatant independent of the osmolality. Since no further release of choline was observed the ratio between extracellular and intracellular choline remained constant at 1:0.7 displaying no osmotic difference.

The amount of PC, one of the main GPC precursors, remained constant between 115 ± 12.1 μmol/g protein at the start of the experiment and 124 ± 11.5 μmol/g protein after 15 h, irrespective of the osmolality used. No significant release of PC into the supernatant could be detected.

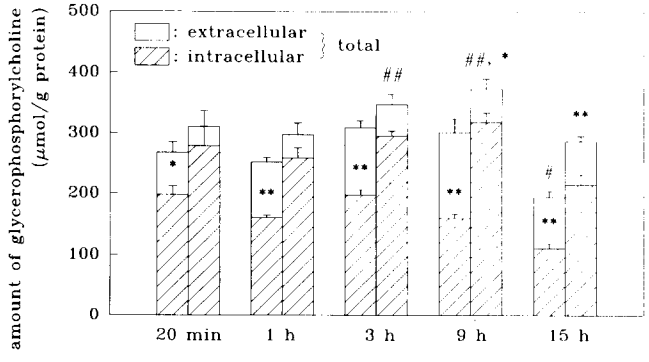

Fig. 1. Effect of osmolality on total amount and release of glycerophosphorylcholine. The total amount composed of intracellular (hatched bars) and released portions (open bars) is given in μmol glycerophosphorylcholine/g protein. The left bars of each time point represent a medium osmolality of 0.3 osmol/l, the corresponding right bars represent 0.9 osmol/l. All values are corrected for unspecific release. Unspecifically released GPC was calculated by taking into account the amount of LDH present in the supernatant. The datapoints represent mean values and the corresponding standard error of mean (S.E.) of six independent experiments performed in duplicate (*, $P < 0.05$; **, $P < 0.005$ for osmotic difference in total content and cellular release; #, $P < 0.01$; ##, $P < 0.001$ for difference with respect to initial amount).

The results obtained for GPC are compiled in Fig. 1. In the beginning total GPC amounted to 269 ± 11 μmol/g protein. In hypotonic medium total GPC, the sum of extra- and intracellular GPC, showed a tendency to increase during the first 9-h incubation, although no statistical significance was reached. After 15 h total GPC was significantly decreased to 72% of the initial value (194 ± 13 μmol/g protein, $P < 0.01$). The distribution between extra- and intracellular GPC changed dramatically with incubation time. After 20 min $26 \pm 3\%$ of total GPC was specifically released from the cells. After 1 h the release amounted to $40 \pm 2\%$ remaining constant for 3 h. After 9 h $48 \pm 2\%$ was released remaining constant for up to 15 h. Cellular GPC release shown in Fig. 1 was corrected for unspecific release taking into account the amount of LDH present in the supernatant. Thus, a rapid initial release of GPC was observed, followed by a phase of slower release until a constant ratio between extra- and intracellular GPC of 0.9:1 was reached. In hypertonic medium total GPC increased significantly. After 3 h GPC amounted to 348 ± 17 μmol/g protein ($P < 0.001$), after 9 h to 374 ± 16 μmol/g protein ($P < 0.001$). After 15 h no significant difference to the initial value could be detected (287 ± 9 μmol/g protein). Concerning the distribution of extra- and intracellular GPC, $10 \pm 4\%$ of the total GPC was specifically released from the cells after 20 min. After 1 h the release amounted to $13 \pm 3\%$, after 3 h to $15 \pm 5\%$ which remained constant for up to 9 h, and after 15 h to $25 \pm 4\%$. The steady-state ratio between extra- and intracellular GPC was 0.33:1. Thus, the amount of GPC released by the cells under hypertonic conditions was reduced compared to hypotonic conditions.

Osmotic influence on glycerophosphorylcholine synthesis from [methyl-^{3}H]choline

Pulse studies. Uptake of [*methyl*-^{3}H]choline into cells was independent of the extracellular osmolality. Within 1 h 192 ± 33 nmol/g protein were incorporated by the cells. After 15 h incorporation amounted to 889 ± 220 nmol/g protein. The same metabolites known from a previous investigation [13] were labeled by [*methyl*-^{3}H]choline namely the phospholipids phosphatidylcholine (PC), lyso PC (LPC) and sphingomyelin (SM) and the water soluble compounds (non-lipids) phosphorylcholine (Pcholine) and GPC. Another water soluble compound remained unidentified. The pulse-time dependence of labeling is shown in Table I. After 1 h significant osmotic differences were found only for PC and GPC. Under hypotonic conditions PC labeling was 1.6-fold lower than under hypertonic conditions (44.8% vs. 71.6%, $P < 0.01$), whereas labeling of GPC was 2.8-fold higher (45.2% vs. 15.9%, $P < 0.05$). After 15 h under hypotonic conditions PC labeling was 2.6-fold lower than under hypertonic conditions (15.8% vs. 41.0%, $P < 0.05$). Labeling of GPC remained higher (68.6% vs. 35.8%, $P < 0.05$). Also Pcholine labeling displayed significant osmotic differences after 15 h. It amounted hypotonically to 4.4% compared to 21.8% under hypertonic conditions. No other metabolite showed any significant osmotic differences in radioactive labeling. Since GPC content was higher under hypertonic conditions the specific activities were also compared (Fig. 2). For choline and PC osmotic differences in specific activity were not significant. After pulsing for 1 h the specific activity of GPC was, however, significantly higher under hypotonic conditions by a factor of 3.1 (14000 vs. 4500 dpm/nmol, $P < 0.05$) and by a factor of 3.4 after 15 h (194200 vs. 56900 dpm/nmol, $P < 0.05$). A significant increase in specific activity with pulse-time, independent of the osmolality, was found for PC ($P < 0.01$) and GPC ($P < 0.05$), the increase being highest for GPC. Considering the cellular GPC (Fig. 1) these data demonstrate a reciprocal proportionality between radioactive labeling and intracellular amount of GPC, suggesting a higher turnover of GPC at low osmolalities.

TABLE I

Pulse labeling of choline metabolites with [methyl-^{3}H]choline: effect of extracellular osmolality

The sum of radioactivity of all labeled metabolites equals 100%. The radioactivity of each labeled metabolite is expressed as percentage. The choline concentration in the medium amounted to 165 nM at a specific activity of 2.81 GBq/nmol. The datapoints represent mean values and the corresponding standard error of mean (S.E.) of four independent experiments performed in duplicate (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$ for osmotic difference).

Metabolite	1-h pulse		15-h pulse	
	0.3 osmol/l	0.9 osmol/l	0.3 osmol/l	0.9 osmol/l
Choline	0.25 ± 0.08	0.20 ± 0.06 n.s.	0.09 ± 0.05	0.17 ± 0.06 n.s.
Pcholine	1.3 ± 0.19	3.1 ± 0.8 n.s.	4.4 ± 0.6	21.8 ± 1.9 **
PC	44.8 ± 11.9	71.6 ± 10.3 **	15.8 ± 7.1	41.0 ± 11.5 *
LPC	0.24 ± 0.04	0.28 ± 0.05 n.s.	0.14 ± 0.03	0.28 ± 0.05 n.s.
GPC	45.2 ± 10.5	15.9 ± 9.1 *	68.6 ± 19.0	35.8 ± 9.7 *
SM	0.14 ± 0.01	0.13 ± 0.01 n.s.	1.2 ± 0.3	1.8 ± 0.74 n.s.
'?'	7.0 ± 1.4	2.1 ± 0.47 n.s.	1.8 ± 0.2	0.81 ± 0.11 n.s.

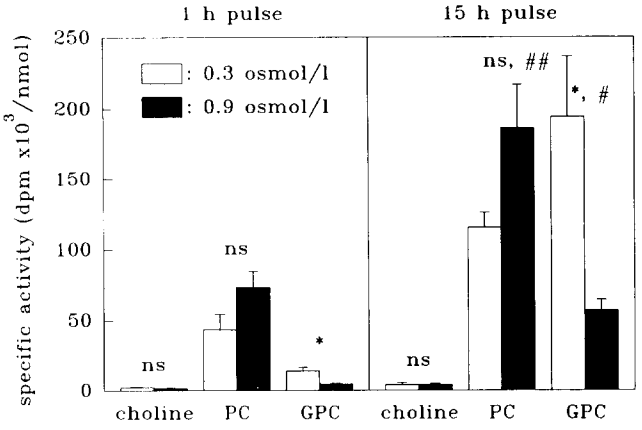

Fig. 2. Specific activity of choline, PC and GPC in pulse experiments with [*methyl*-^{3}H]choline: effect of extracellular osmolality. All values are given in dpm/nmol, the values for choline are multiplied by a factor of 10. The choline concentration in the medium amounted to 165 nM at a specific activity of 2.81 GBq/nmol. The data represent mean values and S.E. of four independent experiments performed in duplicate (n.s., not significant; *, $P < 0.05$ for osmotic difference; #, $P < 0.05$; ##, $P < 0.01$ for difference with pulse time).

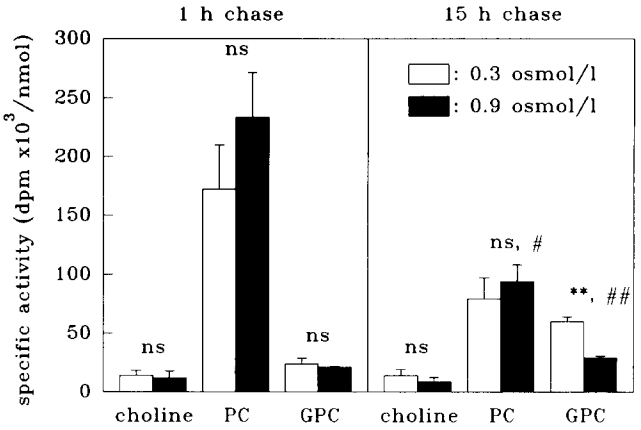

Fig. 3. Specific activity of choline, PC and GPC in pulse-chase experiments with [*methyl*-^{3}H]choline: effect of extracellular osmolality. All values are given in dpm/nmol. The choline concentration in the pulse medium (0.9 osmol/l) amounted to 650 nM at a specific activity of 2.81 GBq/nmol. After a 30-min pulse [*methyl*-^{3}H]choline was replaced by nonlabeled choline at a concentration of 200 μM. The data represent mean values and S.E. of four independent experiments performed in duplicate (n.s., not significant; **, $P < 0.0025$ for osmotic difference; #, $P < 0.01$; ##, $P < 0.005$ for difference with chase time).

Pulse-chase studies. In order to determine the step(s) at which the regulation of GPC turnover occurs pulse-chase experiments were performed. Within the 30-min pulse 401 ± 72 nmol/g protein were incorporated by the cells. The chase time dependence of labeling is shown in Table II. After 1 h no significant osmotic differences were found for any metabolite. However, after 15 h significant osmotic differences for GPC and Pcholine but not for choline and PC were observed. Under hypotonic conditions the amount of radioactivity incorporated into GPC was 1.5-fold higher than under hypertonic conditions (40.3% vs. 26.1%, $P < 0.05$). Labeling of Pcholine was decreased 1.9-fold under hypotonic conditions (11.2% vs. 21.0%, $P < 0.01$). None of the other metabolites investigated were affected by the difference in osmolality. A comparison of the specific activities is shown in Fig. 3. After 1 h specific activities of choline and PC displayed no significant osmotic differences which was also valid for GPC. However, after 15 h the specific activity of GPC under hypotonic conditions was significantly higher by a factor of 2.1 compared to hypertonic conditions (60 200 vs. 29 100 dpm/nmol, $P < 0.0025$). With chase time the specific activities of PC and GPC were significantly altered independent of the osmolality. The specific activity of PC decreased ($P < 0.01$) whereas those of GPC increased ($P < 0.005$). Since in pulse as well as in pulse-chase experiments the specific activity of PC displayed no significant osmotic differences it may be concluded that GPC synthesis from PC is not affected by the osmolality. On the other hand GPC degradation seems to be higher under hypotonic conditions.

TABLE II

Pulse-chase labeling of choline metabolites by [methyl-^{3}H]choline: effect of extracellular osmolality

The sum of radioactivity of all labeled metabolites equals 100%. The radioactivity of each labeled metabolite is expressed as percentage. The choline concentration in the medium (0.9 osmol/l) amounted to 165 nM at a specific activity of 2.81 GBq/nmol during the 30-min pulse. In the chase the choline concentration amounted to 200 μM. The datapoints represent mean values and S.E. of four independent experiments performed in duplicate (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$ for osmotic difference).

Metabolite	1-h chase			15-h chase		
	0.3 osmol/l	0.9 osmol/l		0.3 osmol/l	0.9 osmol/l	
Choline	2.7 ±0.76	3.2 ±1.7	n.s.	7.1 ±2.2	6.1 ±2.5	n.s.
Pcholine	6.6 ±2.0	9.5 ±2.1	n.s.	11.2 ±1.1	21.0 ±3.2	**
PC	63.7 ±7.3	63.7 ±5.9	n.s.	38.1 ±5.8	41.6 ±5.1	n.s.
LPC	0.46±0.11	0.43±0.06	n.s.	0.24±0.05	0.39±0.11	n.s.
GPC	22.6 ±4.9	17.2 ±2.7	n.s.	40.3 ±6.9	26.1 ±3.1	*
SM	0.21±0.03	0.17±0.03	n.s.	1.5 ±0.23	1.5 ±0.33	n.s.
'?'	4.3 ±0.35	4.4 ±0.75	n.s.	1.1 ±0.55	1.9 ±0.5	n.s.

TABLE III

Specific activity of choline, PC and GPC after pulsing with phosphatidyl[methyl-^{3}H]choline: effect of extracellular osmolality

All values are given in dpm/nmol. The PC concentration in the medium (0.9 osmol/l) amounted to 200 nM at a specific activity of 1.11 GBq/nmol. The datapoints represent mean values and S.E. of four independent experiments performed in duplicate (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$ for osmotic difference).

Metabolite	1-h pulse		15-h pulse	
	0.3 osmol/l	0.9 osmol/l	0.3 osmol/l	0.9 osmol/l
Choline	5215 ± 1200	2850 ± 795 **	10110 ± 7875	9735 ± 5770 n.s.
PC	141025 ± 19495	126280 ± 17950 n.s.	157260 ± 28225	157260 ± 68550 n.s.
GPC	3385 ± 1095	2175 ± 720 *	31960 ± 2575	23870 ± 870 **

Osmotic influence on glycerophosphorylcholine synthesis from phosphatidyl[methyl-^{3}H]choline

Pulse studies. Osmotic differences in the reaction sequence from choline to PC including PC degradation were not evident or not significant using [*methyl*-^{3}H]choline. This led to the assumption that GPC synthesis was independent of the extracellular osmolality. To focus on the essential reactions of GPC metabolism where prominent osmotic differences could be found, experiments with phosphatidyl[*methyl*-^{3}H]choline were performed omitting the enzymatic steps of the 'Kennedy pathway' from choline to PC. At a PC concentration of 200 nM the incorporation was linear during 1 h. Labeled metabolites (in addition to PC) were the lipid LPC and the non-lipids GPC and choline. The pulse time dependence of the specific activities is shown in Table III. After 1 h choline had a significantly higher specific activity under hypotonic compared to hypertonic conditions (5215 vs. 2850 dpm/nmol, $P < 0.025$) but after 15 h no significant difference could be seen. With pulse time the specific activity of choline showed no significant alteration. The specific activity of PC displayed no significant differences with pulse time or with osmolality. However, for GPC significant osmotic differences were found. After 1 h the specific activity under hypotonic conditions was increased 1.6-fold compared to hypertonic conditions (3385 vs. 2175 dpm/nmol, $P < 0.05$). After 15 h the specific activity of GPC was hypotonically increased 1.3-fold (31960 vs. 23870 dpm/nmol, $P < 0.01$). With pulse time the specific activity of GPC increased significantly independent of the osmolality ($P < 0.0025$).

Pulse-chase studies. The chase time dependence of the specific activities is shown in Table IV. Neither choline or PC displayed significant osmotic differences during the chase but the specific activity of PC decreased significantly ($P < 0.05$). On the other hand, the specific activity of choline increased significantly ($P < 0.025$). The increase expressed as percentage of the initial value was significantly depending on the osmolality (247.6% vs. 144.4%, $P < 0.05$). After 1 h no significant differences could be found for GPC but after 15 h the specific activity displayed a significant 2-fold increase under hypotonic compared to hypertonic conditions (12115 vs. 6120 dpm/nmol, $P < 0.05$). Additionally, the specific activity of GPC increased significantly with chase time independent of the osmolality ($P < 0.01$). Since no significant evidence for alternative pathways for GPC synthesis could be found in a previous investigation [13] these results provide evidence that GPC synthesis (from PC) is independent of any osmotic influence. Therefore, the osmotic difference in the specific activity of GPC may be related to an osmotically controlled degradation of GPC which is catalyzed by GPC: choline phosphodiesterase.

Influence of chronic antidiuresis on GPC content and GPC turnover

To investigate whether the obtained results in vitro can be transferred to the in vivo situation, pulse experi-

TABLE IV

Specific activity of choline, PC and GPC in pulse-chase experiments with phosphatidyl[methyl-^{3}H]choline: effect of extracellular osmolality

All values are given in dpm/nmol. The PC concentration in the medium (0.9 osmol/l) amounted to 200 nM at a specific activity of 1.11 GBq/nmol. In the chase no unlabeled PC was supplied. The datapoints represent mean values and S.E. of four independent experiments performed in duplicate (n.s., not significant; *, $P < 0.05$; **, $P < 0.025$ for osmotic difference).

Metabolite	1-h chase		15-h chase	
	0.3 osmol/l	0.9 osmol/l	0.3 osmol/l	0.9 osmol/l
Choline	4620 ± 825	5835 ± 2085 n.s.	11440 ± 3605	8420 ± 2125 n.s.
PC	172955 ± 45680	134590 ± 7730 n.s.	98120 ± 11485	91130 ± 7750 n.s.
GPC	1865 ± 595	1410 ± 390 n.s.	12115 ± 1465	6120 ± 105 *
Increase in choline labeling			247.6 ± 42.0%	144.4 ± 14.2% **

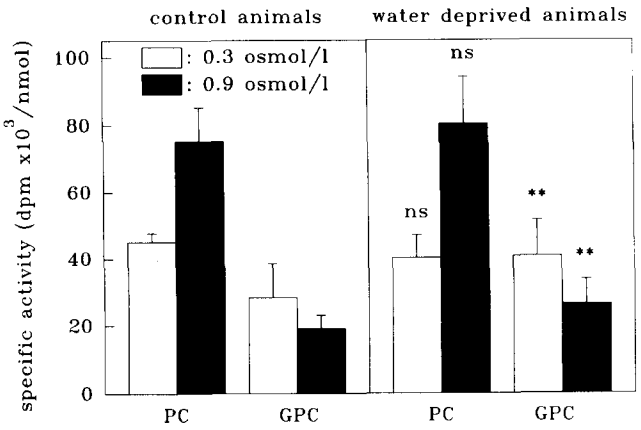

Fig. 4. Specific activity of PC and GPC depending on the diuretic status. Animals were kept without water for 5 days and the antidiuretic period was followed by a pulse of 9 h with 165 nM choline at a specific activity of 2.81 GBq/nmol. All values are given in dpm/nmol. The choline concentration in the medium amounted to 165 nM with a specific activity of 2.81 GBq/nmol. The data represent mean values and S.E. of four independent experiments performed in duplicate (n.s., not significant; **, $P < 0.025$ for difference between water-deprived and control animals).

ments after long-term antidiuresis in vivo were performed. During the antidiuretic period the cellular GPC increased significantly about 45% within 5 days ($P < 0.025$). After isolation the 'antidiuretic' cells were pulsed with [*methyl*-^{3}H]choline for 9 h (Fig. 4). No significant difference to the control concerning the specific activity of PC could be detected. For GPC a significant increase in the specific activity compared to the control was found at 0.3 osmol/l as well as at 0.9 osmol/l which amounted to approximately 40% ($P < 0.025$). After pulsing for 9 h the GPC/PC ratio of the specific activities amounted to 1:1 under hypotonic and 1:3 under hypertonic conditions in antidiuretic samples. In control samples the ratio was hypotonically 1:1.5 and hypertonically 1:4. Since the PC content was unaltered the shift in GPC/PC ratio which is exclusively attributed to an increase in the specific activity of GPC may be caused by increased GPC degradation pointing to GPC: choline phosphodiesterase to be the osmotically controlled enzyme in the adaptation of cellular GPC. This assumption is confirmed viewing GPC content and net synthesis with pulse time in cells of normal and antidiuretic rats (Table V). In 'antidiuretic' samples net synthesis of GPC was significantly lower than in control samples. Therefore, GPC: choline phosphodiesterase seems to be able to switch within hours from low activity under antidiuretic conditions in vivo to high activity under normal conditions (in vitro). Indicated by the significantly lower GPC net synthesis a short-term regulation of the enzyme by extracellular factors seems to be present. Apparently, the in vivo mechanism of increasing cellular GPC seems to be similar to the in vitro mechanism.

Discussion

Isolated inner medullary collecting duct cells of normal rats are capable of synthesis as well as of degradation of the organic osmolyte GPC [11,13]. After elucidation of the reaction sequence generating the organic osmolyte GPC in these cells [13] the regulation of GPC metabolism and herewith regulation of the cellular GPC concentration under different osmotic requirements was of interest. Cellular osmolality adjusted by macromolecules such as proteins, lipids etc., ions such as Na^+, K^+, Cl^- etc., and organic osmolytes such as sorbitol and GPC is regulated by short-term and long-term mechanisms. Volume regulation and release of osmolytes belong to short-term mechanisms [9,12,17, 22]. Thereby release of GPC from IMCD cells is reciprocally proportional to the extracellular osmolality resulting in corresponding alterations in cellular GPC level and thus in cellular osmolality (e.g., Ref. 17). Enzymatic synthesis and degradation of osmolytes belong to long-term mechanisms [18]. Since it seems neccessary that GPC metabolism is coupled to the extracellular osmolality, we attempted to find steps in the enzymatic sequence generating GPC which are influenced by the extracellular osmolality.

Osmotic influence on GPC synthesis and degradation in vitro

In order to ascertain and investigate osmosensitive reactions of GPC metabolism, the use of osmotically

TABLE V

Influence of long-term antidiuresis on total amount of GPC and its increase with pulse time

Rats were deprived from water for 5 days and subsequently pulsed with [*methyl*-^{3}H]choline for 9 h. The choline concentration in the medium amounted to 165 nM at a specific activity of 2.81 GBq/nmol. The values for GPC total and increase with pulse time are given in μmol/g protein. The datapoints represent mean values and S.E. of four independent experiments performed in duplicate (*, $P < 0.025$ for difference in total amount; #, $P < 0.025$; ##, $P < 0.0005$ for difference in GPC increase between antidiuresis and control).

		0-h pulse		9-h pulse	
		0.3 osmol/l	0.9 osmol/l	0.3 osmol/l	0.9 osmol/l
Control	GPC amount	269 ± 7	269 ± 7	311 ± 22	382 ± 16
	Increase			42 ± 15	113 ± 6
Antidiuresis	GPC amount	386 ± 15 *	386 ± 15 *	404 ± 15 *	473 ± 20 *
	Increase			21 ± 14 ##	87 ± 21 #

different conditions was essential. From a previous investigation an extracellular osmolality of 0.9 osmol/l being hypertonic for IMCD cells was useful for elucidation of the reaction sequence generating GPC [13]. For hypotonic conditions an osmolality of 0.3 osmol/l was chosen being high enough to guarantee high cell viability even at extended incubations. These conditions provided a sufficiently large osmotic difference for investigation of osmotic influences on GPC metabolism. Aside from cellular GPC release two different mechanisms could be responsible for the increase in cellular GPC due to increased extracellular osmolality: (1) increase in GPC synthesis and (2) decrease in GPC degradation. To distinguish these mechanisms osmotic differences in specific activities of PC and GPC were of main interest since PC is (via LPC) the direct precursor of GPC.

Experiments with [*methyl*-^{3}H]choline resulted in radioactive labeling (besides choline) of the lipids PC, LPC, and SM and the non-lipids Pcholine, GPC, and an unidentified water-soluble substance. PC had initially the by far highest specific activity in pulse as well as in pulse-chase experiments and displayed no significant osmotic difference under any conditions. The lack of an osmotic influence was also true for choline although its specific activity was lower by a factor of approx. 100. However, GPC being the metabolite with the highest increase in specific activity with pulse time displayed a significant osmotic difference in specific activity being significantly higher under hypotonic conditions although the GPC amount was significantly reduced. The higher specific activity under hypotonic conditions may be based on continuous synthesis of highly radioactive GPC from PC and simultaneous degradation of GPC whose specific activity is (initially) low. Since PC mainly occurs in membraneous compartments which are poorly reactive, a small reactive PC pool for metabolic processes such as GPC synthesis has been proposed [31–33]. Radioactively labeled choline should then cause a high labeling of the metabolic PC pool. Without major exchange with the stable membraneous compartment the radioactivity is subsequently transferred to GPC by phospholipase A_2. Since the GPC pool is approximately 3 times higher than the PC pool, apparently unbound and not compartmented, radioactivity incorporated into GPC from PC is probably much higher than radioactivity released from GPC by GPC:choline phosphodiesterase. This should be true as long as the specific activity of PC is high. Under hypertonic conditions GPC was degraded slowly which caused the amount to be higher and therefore the incorporated radioactivity may be more diluted compared to hypotonic conditions causing the specific activity to be lower. In pulse-chase experiments an extended chase caused the specific activity of GPC which was the only metabolite displaying a significant increase with chase time to be significantly higher under hypotonic conditions. In accordance with pulse experiments GPC synthesis from PC was insensitive to the osmolality assuming the activity of phospholipase A_2 to be similar under different osmotic conditions. By direct measurement of phospholipase A_2 activity in an enzyme assay, Zablocki et al. [21] observed a 30% reduction of total phospholipase A_2 activity in homogenates of MDCK cells adapted to high osmolality. However, since this group found a 70% reduction of GPC:choline phosphodiesterase activity under the same conditions, the relevance of the alteration of phospholipase A_2 activity in intact cells, as determined in the current study, seems to be limited. Assuming that the amount of GPC newly synthesized from PC is equal under different osmotic conditions the significantly different specific activity of GPC, therefore, must have its probable origin in different rates of GPC degradation. Namely, GPC:choline phosphodiesterase seems to have a higher velocity under hypotonic conditions [21]. Short-term pulse experiments as well as the calculation of the specific activity of the intermediate LPC [13] excluded the possibility of de novo synthesis of GPC [34–37] (not shown). Concerning the high Pcholine labeling under hypertonic conditions a feedback inhibition of phosphocholine cytidylyltransferase seems likely. This was proposed by several authors [38–40]. These investigators found evidence that phosphocholine cytidylyltransferase is regulated by the cellular concentration of PC. Although we were unable to detect a significant osmotic difference in the amount of PC a comparably small increase or a reduced turnover may be sufficient to trigger enzyme inhibition. Since the following enzyme choline phosphotransferase is proposed to have a high velocity [13] - no CDP choline could be detected under any conditions - the radioactive label ought to accumulate in Pcholine under hypertonic conditions. Under hypotonic conditions the faster operating enzymes phospholipase A_2 and GPC:choline phosphodiesterase cause a higher turnover without inhibition of phosphocholine cytidylyltransferase resulting in a significantly lower labeling of Pcholine.

Further experiments with phosphatidyl[*methyl*-^{3}H]-choline confirmed the proposal of different osmotic sensitivities of the enzymes phospholipase A_2 and GPC:choline phosphodiesterase. Besides PC labeled metabolites were LPC, GPC, choline and (at extended incubations) Pcholine (not shown). In pulse experiments exclusively the specific activity of GPC displayed significant osmotic differences with the specific activity being higher under hypotonic conditions. Concerning the specific activities of choline and GPC which were much lower than after pulsing with [*methyl*-^{3}H]choline it is likely that radioactive PC applied with the incubation medium is first entering the stable membraneous

PC compartment (see above). As an exchange with the metabolic PC pool is quite low, the GPC/PC ratio must be lower than if radioactive PC is generated in the metabolic pool as in experiments with [*methyl*-^{3}H]choline. A proposed 'channeling' of GPC synthesis from choline – the concerted action of the involved enzymes without release of intermediates [31] – would make it likely that application of [*methyl*-^{3}H]choline – although more enzymatic steps for GPC synthesis are involved – causes a much higher radioactive labeling of GPC. This assumption is in correspondence with the results since in these experiments the GPC/PC ratio of the specific activities was more than 10-times higher after 1 h than in phosphatidyl[*methyl*-^{3}H]choline experiments. After 15 h the ratio decreased to the 7-fold amount indicating that exchange between the stable and the metabolic PC pools was increased. In pulse-chase experiments the increase in specific activity of choline with chase time expressed as percentage of the initial value was significantly higher under hypotonic conditions. This is in good agreement with the assumption that more (radioactive) GPC was degraded by GPC: choline phosphodiesterase under hypotonic conditions. This increased hydrolysis would lead to an augmentation of radioactive choline.

Summarizing the results of the in vitro experiments we found PC degradation by phospholipase A_2 (corresponding to GPC synthesis) to be completely independent of the extracellular osmolality indicating a quite constant GPC synthesis. Although no experiments were performed with radioactive GPC in view of its degradation directly, the interpretation of the data strongly suggests that cellular GPC metabolism is regulated by the osmosensitive enzyme GPC: choline phosphodiesterase which is inhibited in hypertonic surroundings. This hypothesis is consistent with the findings of Ullrich [20] who proposed an inhibitory effect of NaCl and urea on GPC hydrolysis. The results obtained with MDCK cells by Zablocki et al. [21] who found that increase in extracellular osmolality reduced GPC: choline phosphodiesterase activity thus increasing cellular GPC confirm the assumption of NaCl and urea regulating enzyme activity.

Osmotic influence on GPC metabolism in vivo

To examine transferability of the in vitro results to the in vivo situation experiments at normal conditions and at a sustained antidiuresis [12,41] (in vivo) were performed in parallel. Antidiuretic conditions caused a significant increase in cellular GPC probably reflecting an increased net synthesis. However, since in the pulse experiments following the antidiuretic period the specific activity of PC was not different from the control, cellular GPC synthesis seems to be unaltered and a decreased GPC degradation may be likely. This assumption was confirmed by the pulse experiments where the specific activity of GPC displayed a significant increase compared to the control. Probably, the GPC: choline phosphodiesterase was 'switched' from an inhibited state induced by antidiuresis to a more active state because of the reduction in extracellular osmolality. Furthermore, it seems unlikely that during the antidiuretic period a reduction in the number of enzyme molecules or induction [42,43,45–47] of a GPC: choline phosphodiesterase with different properties could take place since the relative osmotic difference in specific activity of GPC was identical in 'normal' cells and cells of antidiuretic animals; the osmotic difference expressed as percentage exactly matched that under normal conditions. This is confirmed by Zablocki et al. [21] who found a 50% reduction of GPC: choline phosphodiesterase activity in MDCK cells within 2 h after the osmolality was increased – a time being very short for genetic adaptation [44]. Therefore, the enzyme is obviously regulated by extracellular factors – NaCl and/or urea have been proposed (see above [20,21]) although the exact nature remains to be determined – which are transformed into intracellular modifiers of the enzyme activity. The importance of an inhibitory mechanism for GPC: choline phosphodiesterase under antidiuretic conditions can be derived from the requirements the organism has to face in vivo. An antidiuretic status for the kidney seems to be the normal status. Thus, if antidiuresis is 'normal', a hypertonically reduced activity of GPC: choline phosphodiesterase would be by far more economic. Short-term hypotonic challenges may be faced by rapid cellular release of GPC which causes the cellular GPC concentration to decrease from initially 27 mM to 14 mM within 1 h. In this way GPC behaves similar to sorbitol which is another organic osmolyte. Its cellular concentration which initially amounts to 13 mM rapidly decreases below 8 mM in hypotonic surroundings [23].

In conclusion we find strong evidence for osmosensitive GPC degradation catalyzed by GPC: choline phosphodiesterase to be responsible for regulation of the cellular GPC concentration in IMCD cells in vitro. On the other hand we find GPC synthesis by phospholipase A_2 to be entirely unaffected by the extracellular osmolality. In addition, the cells display a fast osmotic adaptation in membrane permeability for GPC resulting in a significant release in hypotonic surroundings. In vivo a similar osmoregulation of GPC: choline phosphodiesterase as in vitro seems to exist. No indication of a genetically adapted GPC: choline phosphodiesterase could be detected.

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