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Metabolism of the 'organic osmolyte' glycerophosphorylcholine in isolated rat inner medullary collecting duct cells. I. Pathways for synthesis and degradation

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In isolated inner medullary collecting duct (IMCD) cells the adaptation to changes in extracellular osmolarity involves alterations in intracellular content of organic osmolytes such as glycerophosphorylcholine (GPC), sorbitol and others. To elucidate the basis of such alterations, the metabolism of GPC in IMCD cells was investigated with the labeled GPC precursor [*methyl*-^{3}H]choline. The lipids phosphatidylcholine (PC), lyso PC (LPC) and sphingomyelin (SM), as well as the non lipids phosphorylcholine (Pcholine), GPC and an unknown water-soluble compound could be identified as intermediates of choline metabolism. In pulse-chase experiments the radioactivity of PC expressed as specific activity was at a higher level than the other metabolites (> 10-fold after 1 h). Extended chase incubations caused the specific activity of PC and LPC to decrease significantly. GPC was the only metabolite with a significant increase in specific activity under these conditions, suggesting that PC (via LPC) could be the precursor of GPC. In short-term pulse experiments the specific activity of PC and LPC was always significantly higher compared to the specific activity of GPC. Pulse chase incubations using phosphatidyl[*methyl*-^{3}H]choline showed a significant decrease in specific activity of PC after 15 h accompanied by a significant increase in specific activity of LPC as well as GPC. Inhibition of the PC hydrolyzing enzyme phospholipase A_2 revealed a significant increase in the specific activity of PC. For GPC, a significant decrease in the radioactive labeling could be detected. The total amount of PC decreased by 10% under these conditions whereas the amount of GPC decreased by 22% which was significantly higher because of GPC breakdown. GPC degradation was catalyzed by GPC:choline diesterase generating choline (and phosphoglycerol). Significant activity of GPC:phosphocholine diesterase could not be detected. Betaine synthesis from choline was also not present. The slowest, and probably rate-limiting reaction of GPC synthesis from choline may be the reaction of phosphocholine cytidylyltransferase generating CDP choline, since no radioactive CDP choline could be detected under any conditions. Thus, isolated IMCD cells possess the ability for the synthesis of GPC from choline via PC and LPC, as well as for the GPC degradation to choline (and phosphoglycerol). Significant experimental evidence for the occurrence of de-novo synthesis of GPC from choline or a precursor function of GPC for PC could not be detected. However, although the former possibility seems unlikely, a final proof is still lacking.

Introduction

Due to their location in the renal papilla, IMCD cells are exposed to continuous changes in the osmolarity of their surroundings. Urine osmolarity can vary widely between < 300 and > 2500 mosmol/l depending on the hydration state and the water balance of the organism [1–3]. To counter osmotic challenges, IMCD cells possess various strategies whereby a few cellular organic compounds – organic osmolytes – play an important role. Organic osmolytes such as sorbitol, glycerophosphorylcholine (GPC) and others [4–8] are 'nonperturbing' in the sense that they do not disturb cellular reactions even at high concentrations [9]. Under hypotonic conditions organic osmolytes such as GPC, which was first discovered in the kidney by Ullrich [10], are specifically released by the cells to lower the cellular osmolarity [11,12]. Under hypertonic conditions the release is replaced by several mechanisms, e.g., volume reduction, net synthesis of osmolytes and/or uptake from interstitial compartments, whose importance for intracellular accumulation is not yet fully elucidated. Although the net synthesis of GPC

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is a slow process [13,14], we investigated its cellular metabolism with the aid of known radioactively labeled precursors. From several tissues a part of the metabolic pathway generating GPC is already known: The Kennedy pathway from choline to phosphatidylcholine was first discovered by Kennedy and Weiss [15] in the liver. Recently, the synthesis of GPC from PC and its degradation to choline (and phosphoglycerol) could be demonstrated in Madin Darby canine kidney (MDCK) cells [16], whole renal medulla [17] and a variety of other cell types [18]. In addition, de-novo synthesis of GPC via various pathways has been postulated [19–22], although those results are discussed controversially [18,23–25]. The pathways of GPC metabolism in the collecting duct cells per se are, however, essentially unknown. This paper focuses on the ability of IMCD cells to metabolize GPC employing in-vitro incubations of a pure fraction of these cells. Our main interest was the elucidation of the pathway(s) for GPC synthesis and degradation. A further aim was to find rate-limiting reactions which may serve as a control for the whole reaction sequence. In addition this investigation should provide the framework to study mechanisms of regulation during changes in extracellular osmolarity since an osmotic influence on GPC metabolism seems neccessary: The need of organic osmolytes ought to be higher in hypertonic compared to hypotonic surroundings. This is the main subject of a forthcoming paper.

The studies presented in this article are part of the Ph.D. thesis of H.G. Bauernschmitt and have been published in abstract form [26].

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 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) [27]. After mincing the tissue, the pieces were incubated in the same buffer with addition of 0.2% collagenase, 0.2% hyaluronidase and 0.001% DNase at 37°C gassing with room air for 75 min. IMCD cells were isolated by repeated low-speed differential centrifugation as described previously [28]. The final cell preparation contained > 95% collecting duct cells. To investigate the GPC metabolism, the osmolarity of the buffer was elevated to 0.9 osmol/l by adding NaCl to a final concentration of 418 mM during the experiments. In one set of experiments the buffer osmolarity was lowered to 0.3 osmol/l by reducing NaCl to a final concentration of 118 mM. All incubations were at 37°C for the time periods given in the Results section. The viability of IMCD cells was routinely checked by the determination of lactate dehydrogenase activity in the cell supernatants [27]. For up to 3 h, cell viability was greater than 95% followed by a linear decrease to 85% after 15 h. The cell viability after 9 h of incubation with quinacrine ranged at 88% which was not significantly different from control incubations.

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 were added. The PC concentration amounted to 200 nM. In pulse experiments the radioactive precursor was introduced to the cells for the indicated time periods (up to 15 h). In pulse-chase experiments the radioactive precursor was removed after a pulse of 30 min by sedimenting the cells for 3 min at $28 \times g$ at 4°C. The cells were then washed once with 1 ml Hepes buffer using the same centrifugation procedure. This was followed by resuspension of the cells in the desired chase medium. In [*methyl*-^{3}H]-choline experiments the concentration of the 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 were performed in a volume of 800 μl (containing approx. 0.4 mg of protein). At the end of each incubation the cells were separated from the incubation medium by a centrifgation for 10 min at $1500 \times g$ at 4°C. This was done to analyse the labeled cellular and extracellular metabolites separately. Samples were analysed either immediately or after storage at −20°C.

Lipid extraction. Lipids were extracted from the cells and the supernatants using methanol/trichloromethane/water (1:1:1) according to the method of Bligh and Dyer [29]. After separation of the two layers the solvent of each sample was evaporated in a vacuum centrifuge. The pellets containing lipids, respectively, non-lipids were resuspended in a small volume of trichloromethane/methanol (1:1) or water.

Thin-layer chromatography. For water-soluble metabolites a system consisting of methanol, 0.5% NaCl and ammonia (100:100:4) was used (modified after Refs. 30 and 31). Lipid metabolites were separated in a system with the components trichloromethane, methanol, acetic acid and water (100:39:3:6) (modified after Ref. 32). Silica gel 60-plates (Merck, Darmstadt, Germany) were used for both systems. Development of the chromatograms took between 1.5 and 2 h before being dried for at least 30 min in a cold air stream. For the identification of the labeled metabolites, pure standards were run on the same chromatograms and stained with iodine vapor comparing the R_f values with those

obtained in experiments with radioactive choline and PC.

Autoradiography. The dried chromatograms were placed in the measuring chamber of a digital autoradiograph (Berthold, Bergisch-Gladbach, Germany). After 2 h at a voltage of 1800 V in a saturated methylal atmosphere in argon/methane (90:10) at 3°C, the specific signals emitted from the radioactive spots differed sufficiently from the nonspecific background. The radioactivity of each metabolite was measured as cpm. To transform cpm into dpm values, radioactive standards were used and the original radioactivity applied was related to the measured radioactivity. The factors obtained were used to correct for quench. In all experiments the recovery of the radioactivity initially applied to the chromatographic plate amounted to 94 ± 4%.

Determination of choline, phosphatidylcholine and glycerophosphorylcholine. GPC was determined by the method of Wirthensohn and Guder [33]. The procedure is based on the 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 [33] in non-hydrolysed samples. The principle for the measurement of PC is the hydrolysis to phosphorylcholine by phospholipase C followed by the dephosphorylation of phosphorylcholine to choline by alkaline phosphatase. After inactivation of these enzymes the method is identical with the method for choline determination. A commercially available test kit was employed for PC determination (Boehringer-Mannheim, Mannheim, Germany).

Determination of lysophosphatidylcholine. LPC was determined by performing the iodine-starch reaction directly on developed chromatographic plates and densitometry with an Ultroscan XL laser densitometer (LKB, Uppsala, Sweden). LPC standards and samples from lipid extracts were stained with iodine vapor for 24 h, put into an air stream for 5 min to remove nonspecifically-bound iodine and subsequently dipped into a solution of 10% (w/v) potato starch/potassium iodide for 5 s followed by excessive washing with demineralized water. The light blue stained plates with dark blue spots marking LPC, as well as other lipids were dried at 40°C after which they could be measured either immediately or within 7 days without fading in spot intensity. The standardized procedure allowed the quantification of LPC by comparing the spot intensity of the LPC standards with the intensity of LPC from lipid extracts.

Protein determination. Protein was measured in triplicate according to Lowry et al. [34]. Samples 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.) obtained with the computer program EXCEL 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. Phosphatidyl[*methyl*-^{3}H]choline (L-α-dipalmitoyl; 1.11 TBq/mmol) was supplied from NEN Dupont. The labeled compounds were checked for purity by thin-layer chromatography. Both compounds migrated as single spots, indicating the lack of radioactive contaminants. All chemicals were of highest purity commercially available and were purchased from Sigma, Boehringer-Mannheim, Fluka or Riedel-de Haen unless otherwise stated.

Results

Total amount of choline, phosphatidylcholine, lysophosphatidylcholine and glycerophosphorylcholine

Following cell isolation at 0.6 osmol/l, incubations were performed at an osmolarity of 0.9 osmol/l. No choline was added in these experiments. The total amounts of GPC, PC, LPC and choline were calculated by adding the values obtained for the cells and the corresponding supernatants. At the start of the experiment the GPC content amounted to 269 ± 11 μmol/g protein. The choline, PC and LPC content was 56 ± 6, 115 ± 12 and 1.5 ± 0.83 μmol/g protein, respectively. No significant change could be observed after 1 h but after 15 h choline had significantly increased about threefold (190 ± 23 μmol/g protein, $P < 0.005$) whereas GPC (287 ± 13 μmol/g protein), PC (124 ± 12 μmol/g protein) and LPC showed no significant change. Long-term experiments with high extracellular GPC revealed no uptake from the medium (data not shown).

Metabolism of [methyl-^{3}H]choline

Uptake. We examined the uptake of the GPC precursor choline into IMCD cells. In pulse experiments of up to 1 h, a linear relationship between incubation time and cellular uptake of [*methyl*-^{3}H]choline could be seen. After 1 h, linearity was no longer observed due to the decrease of the extracellular precursor concentration. At a choline concentration of 165 nM the cells incorporated 192 ± 33 nmol/g protein within 1 h and after 15 h the incorporation amounted to 889 ± 220 nmol/g protein. In pulse-chase experiments the cells incorporated 401 ± 72 nmol/g protein after a pulse of 30 min. Quantitative studies at various choline

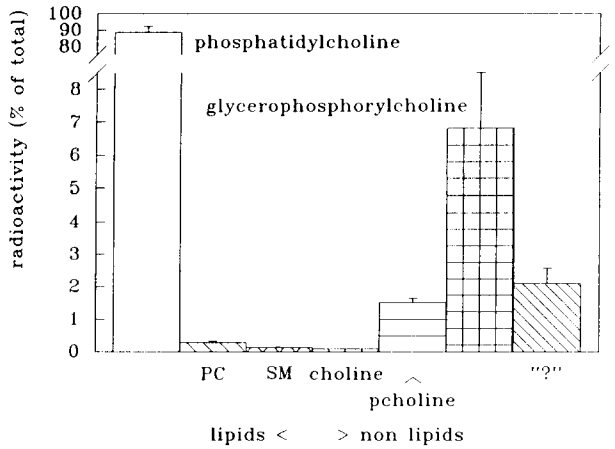

Fig. 1. Distribution of radioactivity after pulsing with [*methyl*-^{3}H]choline. The sum of radioactivity of all labeled metabolites equals 100%. The radioactivity of each labeled metabolite is expressed as percentage. The pulse was performed for 1 h with 165 nM choline with a specific activity of 2.81 GBq/nmol at a medium osmolarity of 0.9 osmol/l. The datapoints represent mean values and the corresponding standard error of mean (S.E.) of four independent experiments.

concentrations revealed a maximum velocity of 58 μmol/g protein per h for the uptake (data not shown). This is in close agreement to results found for IMCD cells by Bevan and Kinne [35].

Pulse studies. [*methyl*-^{3}H]Choline was incorporated into several cellular choline-containing metabolites (Fig. 1). These could be identified as the phospholipids PC, LPC and SM and the water-soluble compounds (non-lipids) Pcholine and GPC. Another water-soluble compound remained unidentified. No radioactively-labeled betaine could be detected.

After 1 h 89% of the total incorporated radioactivity was found in PC which is one of the most abundant membrane lipids. Other metabolites such as GPC and Pcholine were labeled to a much lesser extent (GPC 7%; Pcholine 1.5%). Metabolites such as LPC and SM were weakly labeled (about 1%). The unidentified substance was initially highly labeled (7.5 min, 26.6%) but quickly lost its radioactivity (2% after 1 h). The calculation of specific activities allowed for the correction of different intracellular concentrations of labeled metabolites. After 1 h 104 μmol/g protein PC and 298 μmol/g protein GPC were found. The high radioactive labeling of PC in combination with the (relatively) small pool size caused the specific activity of PC to be by far the highest compared to GPC and choline (PC, 73075 dpm/nmol; GPC, 4470 dpm/nmol; choline, 160 dpm/nmol). The high labeling of PC after 1 h may mean that the phospholipid has an important role in the metabolism of choline and GPC.

Short-term pulse studies. To discriminate between (initial) de-novo synthesis of GPC and synthesis via the Kennedy pathway followed by successive hydrolysis of PC pulse experiments at shorter time points were performed. Table I shows the specific activities of PC, LPC and GPC during 30 min. After 7.5 min PC already had a significantly higher specific activity than GPC (5160 vs. 2180 dpm/nmol, $P < 0.01$). The specific activity of LPC ranged between those of PC and GPC displaying a significant difference to that of PC and GPC (2650 dpm/nmol, $P < 0.05$). During the pulse the specific activities of all metabolites increased linearly showing the highest increase for PC and LPC. Since the specific activities of PC and LPC significantly exceeded that of GPC, the experiment revealed no evidence for de-novo synthesis of GPC (and synthesis of PC from GPC via LPC which is termed acyl-GPC by Infante [19]), although the possibility of de-novo synthesis could not be ruled out completely because of the initially decreasing ratios of GPC/PC.

Pulse-chase studies. The pulse-chase studies were expected to reveal the sequence of reactions since [*methyl*-^{3}H]choline was removed after a pulse of 30 min. The chase-time-dependence is shown in Fig. 2. As already shown in the pulse experiments, the bulk of radioactivity was initially associated with PC (1 h, 63.7%). Extended chase incubations led to a significant decrease for PC (15 h, 41.6%, $P < 0.01$), as well as for the unidentified substance (1 h, 4.4%; 15 h, 1.9%, $P < 0.05$). The radioactivity of other metabolites such

TABLE I

Specific activity of PC, LPC and GPC: short-term pulse with [methyl-^{3}H]choline

The values for PC, LPC and GPC are given in dpm/nmol. The choline concentration in the pulse 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. The difference between PC and GPC was always significant at $P < 0.01$ (* $P < 0.05$; ** $P < 0.01$ for differences between LPC and PC, or LPC and GPC).

Lipid	Pulse duration (min)		
	7.5	15	30
PC	5160±205 **	16040±1090 **	35850±3270 **
LPC	2650±235	4290± 345	8750±1575
GPC	2180±175 *	2345± 100 **	3570± 355 **
Ratio GPC/PC	1:2.4± 0.2	1:6.9± 0.7	1:10.3± 1.8

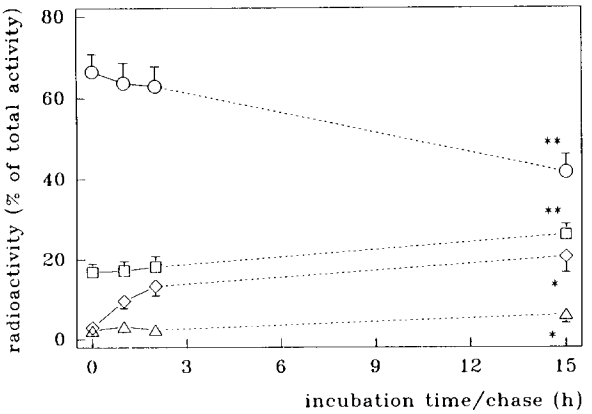

Fig. 2. Pulse-chase incubation with [*methyl*-^{3}H]choline. The sum of radioactivity of all labeled metabolites equals 100%. The radioactivity of each labeled metabolite is expressed as percentage (circles, PC; squares, GPC; triangles, choline; diamonds, Pcholine). The choline concentration in the pulse medium (0.9 osmol/l) amounted to 650 nM with a specific activity of 2.81 GBq/nmol. In the chase the concentration of unlabeled choline was 200 μM. The datapoints represent mean values and the corresponding S.E. of four independent experiments (* $P < 0.05$; ** $P < 0.01$ for difference between 1 and 15 h).

as GPC, choline and Pcholine however increased significantly (GPC, 1 h, 17.2%; 15 h, 26.1%, $P < 0.01$; choline, 1 h, 3.2%; 15 h, 6.1%, $P < 0.05$; Pcholine, 1 h, 9.5%; 15 h, 21.0%, $P < 0.05$). Metabolites such as LPC and SM were weakly labeled (1%) with minor changes over chase time. From the strong increase at extended incubations it may be concluded that GPC and Pcholine are likely 'end products' of the choline metabolism, whereas PC may be an intermediate (Table II).

As the amount of radioactivity in the chase was constant, the calculation of the specific activities showed the transfer of label from one metabolite to the other (Table III). Initially, PC had a specific activity far exceeding those of LPC, GPC and choline (1 h, PC, 233490 dpm/nmol; LPC, 112735 dpm/nmol; GPC, 20890 dpm/nmol; choline, 1195 dpm/nmol). Confirming assumptions that PC may be a quickly labeled intermediate, its specific activity decreased significantly with the chase time (15 h, 60% decrease compared to 1 h, $P < 0.01$) which was also true for LPC (15 h, 50% decrease compared to 1 h, $P < 0.01$). Correspondingly, the specific activity of GPC increased significantly (15 h, 39% increase compared to 1 h, $P < 0.005$). The

TABLE II

Labeling of choline metabolites: chase-time-dependence after pulsing with [methyl-^{3}H]choline

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 at least four independent experiments (* $P < 0.05$; ** $P < 0.01$ for difference between 1 h and 15 h).

Metabolite	Chase time (h)			
	0	1	2	15
Choline	2.2 ±0.7	3.2 ±1.7	2.4 ±0.9	6.1 ±2.5 *
Pcholine	2.9 ±0.6	9.5 ±2.1	13.3 ±2.8	21.0 ±3.1 *
PC	66.6 ±5.0	63.7 ±5.9	62.9 ±5.9	41.6 ±5.1 **
LPC	0.43±0.07	0.43±0.06	0.46±0.09	0.39±0.11
GPC	16.9 ±2.4	17.2 ±2.7	17.9 ±3.2	26.1 ±3.1 **
SM	0.09 ±0.03	0.17 ±0.03	0.37 ±0.09	1.5 ±0.33
'?'	11.3 ±1.7	4.4 ±0.75	3.3 ±0.7	1.9 ±0.50 *

TABLE III

Specific activity of choline, PC, LPC and GPC: chase-time-dependence after pulsing with [methyl-^{3}H]choline

The values for choline, PC, LPC and GPC are given in dpm / nmol. The difference after 15 h is indicated in percent of the 1 h value. The choline concentration in the pulse medium amounted to 650 nM at a specific activity of 2.81 GBq / nmol. In the chase the [methyl-^{3}H]choline was replaced by unlabeled choline at a concentration of 200 μM. The data represent mean values and S.E. of four independent experiments (n.s., not significant; * $P < 0.01$; ** $P < 0.005$ for difference between 1 and 15 h).

Metabolite	Chase time (h)				Difference 1/15 h
	0	0.5	1	15	
Choline	1270± 345	1975± 970	1195± 575	875± 345	−27%, n.s.
PC	217390±42745	245285±42365	233490±38005	93750±14290	−60% *
LPC	91035±22450	98315±29215	112735±41600	56165±12205	−50% *
GPC	20075± 2070	20725± 305	20890± 705	29095± 1370	+39% **
Ratio GPC/PC	1:9.8± 2.4	1:11.8± 2.0	1:11.3± 2.0	1:3.3± 0.5	+206% *

TABLE IV

Labeling of PC metabolites: chase-time-dependence after pulsing with phosphatidyl[methyl-^{3}H]choline

The sum of radioactivity of all labeled metabolites equals 100%. The radioactivity of each labeled metabolite is expressed as percentage. The PC concentration in the medium (0.9 osmol/l) amounted to 200 nM at a specific activity of 1.11 GBq/nmol during the 30-min pulse. In the chase no unlabeled PC was supplied. The datapoints represent mean values and the S.E. of at least three independent experiments (* $P < 0.05$; ** $P < 0.025$ for difference between 1 and 15 h).

Metabolite	Chase time (h)			
	0	1	2	15
PC	94.8 ±0.9	94.2 ±0.9	93.3 ±0.8	80.8±3.5 **
LPC	0.64±0.18	0.83±0.21	0.93±0.25	1.1±0.30
GPC	1.5 ±0.5	2.9 ±0.9	3.2 ±0.8	8.3±2.6 **
Choline	2.7 ±0.6	1.6 ±0.2	1.8 ±0.2	8.6±0.9 **
Pcholine	0.33±0.1	0.45±0.1	0.47±0.1	1.7±0.5 *

GPC/PC ratio was 1:11.3 after 1 h. Within 15 h the ratio increased significantly to 1:3.3 ($P < 0.01$), showing that extended incubations strongly favor the labeling of GPC over that of PC. The above results show that the synthesis of PC from [*methyl*-^{3}H]choline is (at least initially) a fast reaction compared to its degradation. These data are also consistent with the view that PC may be the precursor of GPC (via LPC) due to the corresponding increase in the specific activity of GPC compared to the decrease in PC and LPC. PC may also be the precursor of Pcholine. This might be concluded from the significant increase in the radioactive Pcholine labeling ($P < 0.05$) which occurred during an extended incubation (labeling of PC decreased).

In parallel experiments with [*alkyl*-^{14}C]choline where the C-skeleton instead of methyl groups was radioactively labeled, identical results were obtained. This indicated that all labeled compounds may be related to the transfer of [*methyl*-^{3}H]choline and not to a transfer of [^{3}H]methyl groups.

Metabolism of phosphatidyl[methyl-^{3}H]choline

In order to concentrate on the GPC synthesizing and degrading reactions, we shortened the reaction cascade and reduced the number of labeled metabolites by the use of phosphatidyl[*methyl*-^{3}H]choline. After pulsing for 1 h, labeled metabolites were the lipid LPC and the non-lipids GPC and choline. However, the velocity of metabolism was much slower than that of [*methyl*-^{3}H]choline.

Table IV shows pulse-chase experiments at a 30-min pulse period. Extended chase incubations led to a significant decrease in the labeling of PC (1 h, 94.2%; 15 h, 80.8%, $P < 0.025$). The radioactivity of other metabolites increased (e.g., GPC, 1 h, 2.9%; 15 h, 8.3%, $P < 0.025$). However, the decrease in PC labeling with the chase time was slow, possibly because of the minor availability of the added radioactive PC (see below). Table V shows the specific activity of choline, PC, LPC and GPC. After 1 h, PC had the highest specific activity (PC, 134590 dpm/nmol; LPC, 83590 dpm/nmol; GPC, 1410 dpm/nmol; choline, 5835 dpm/nmol). An extended chase caused the specific activity of PC to decrease significantly (15 h, 32% decrease compared to 1 h, $P < 0.025$). A significant increase in the specific activity was found for LPC (15 h, 52% increase compared to 1 h, $P < 0.025$), GPC (15 h, 334% increase compared to 1 h, $P < 0.025$) and for choline (15 h, 56% increase compared to 1 h, $P < 0.005$). The GPC/PC ratio showed a significant increase from 1:120.1 after 1 h to 1:14.9 after 15 h ($P < 0.025$). Extended chase incubations with phos-

TABLE V

Specific activity of choline, PC, LPC and GPC: chase-time-dependence after pulsing with phosphatidyl[methyl-^{3}H]choline

The values for choline, PC, LPC and GPC are given in dpm/nmol. The PC concentration in the pulse medium amounted to 200 nM at a specific activity of 1.11 GBq/nmol. In the chase no unlabeled PC was supplied. The data represent mean values and S.E. of three independent experiments (n.s., not significant; * $P < 0.025$; ** $P < 0.005$ for difference between 1 and 15 h).

Metabolite	Chase time (h)			Difference 1/15 h
	0	1	15	
PC	147825±17390	134590± 7730	91130± 7750	−32% *
LPC	73775±10055	83560±14550	126665±11390	+52% *
GPC	890± 205	1410± 390	6120± 105	+334% *
Choline	8450± 1045	5835± 2085	8420± 2125	+56% **
Ratio GPC/PC	1:188.2± 54.8	1:120.1± 45.9	1:14.9± 1.0	*

TABLE VI

Influence of phospholipase A_2 inhibition on the labeling of choline metabolites

The radioactivity of each labeled metabolite is expressed as percentage of the total incorporated radioactivity. Additionally, the radioactivity of PC and GPC is given as specific activity in dpm/nmol. Pulse incubations were performed for 9 h. The choline concentration in the medium (0.9 osmol/l) amounted to 165 nM at a specific activity of 2.81 GBq/nmol. The concentration of quinacrine amounted to $5 \cdot 10^{-5}$M/sample. The data represent mean values and S.E. of eight independent experiments (n.s., not significant; * $P < 0.025$; ** $P < 0.005$ for difference between control and quinacrine samples; *** $P < 0.005$ for difference between decrease in PC and GPC).

	Control (%)	Amount (μmol/g protein)	Quinacrine (%)	Amount (μmol/g protein)
Choline	0.27 ± 0.02	124.0 ± 13.8	0.31 ± 0.07, n.s.	142.0 ± 10.9 (+ 14.9%)
Pcholine	18.9 ± 2.8		14.3 ± 2.4, n.s.	
PC	33.4 ± 4.3	122.9 ± 5.2	38.6 ± 2.8 *	110.8 ± 10.8 (− 10.0% *)
GPC	50.6 ± 5.5	352.1 ± 11.8	47.9 ± 5.1 **	277.2 ± 23.0 (− 22.3% **,***)
PC (specific activity (dpm/nmol))	78845 ± 9555		100845 ± 6475 *	
GPC (specific activity (dpm/nmol)	41685 ± 6005		50150 ± 6975, n.s.	

phatidyl[*methyl*-^{3}H]choline lead to a shift in the GPC/PC ratio favoring GPC. Interestingly, only a small initial labeling of Pcholine could be detected in these experiments (0.45% after 1 h) indicating that the degradation of GPC is nearly exclusively catalyzed by a diesterase rather than a phosphodiesterase. The small labeling of Pcholine also does not support the assumption that PC is the direct precursor of Pcholine.

Influence of inhibited phosphatidylcholine degradation on the GPC / PC ratio

The inhibition of phospholipase A_2, which is responsible for the hydrolysis of PC, should give further proof for PC being the precursor of GPC. A 9 h pulse experiment in the presence of $5 \cdot 10^{-5}$ M quinacrine is shown in Table VI. A significant increase in the radioactivity associated with PC (38.6% vs. 33.4% at control, $P < 0.025$) and a significant decrease in that of GPC (47.9% vs. 50.6% at control, $P < 0.005$) in the presence of quinacrine could be seen. However, the total amount of PC decreased under these conditions (10%, $P < 0.025$). The amount of GPC decreased also (22.3%, $P < 0.01$) but the decrease in GPC was significantly higher than the decrease in PC ($P < 0.005$). This caused the specific activity of PC to increase significantly whereas that of GPC displayed no significant difference. Since the amount of choline increased by 15% ($P < 0.025$) quinacrine seems to inhibit the turnover of choline generally due to other effects on cellular metabolism. The differences in the specific activity of PC and GPC compared to control experiments are probably due to (partial) prevention of PC hydrolysis to GPC by inhibition of phospholipase A_2.

Discussion

The IMCD cells used for the present investigation have been shown to be a useful model system for the elucidation of various physiological and biochemical processes [27,28,36–38]. In primary culture they show numerous properties of native cells [39]. Comparing tissue homogenates [6], isolated tubuli [4,40,41] and isolated IMCD cells [27,36,37], no functional or physiological differences could be found. However, the presented in-vitro experiments reveal the capabilities of isolated cells but whether the cellular behaviour is the same in vivo as in vitro remains to be established. An interesting feature showing a difference between in vivo and in vitro is the complete lack of betaine synthesis: Under no experimental conditions could a radioactive labeling of betaine be measured (see below).

For the synthesis of GPC several reaction sequences have been discussed [18–25,78]. A widespread pathway found in many different tissues is leading from choline via PC and LPC to GPC whereby the first part (choline to PC) is called the Kennedy pathway [15,42–45]. Successive cleavage of the fatty-acid residues of PC by phospholipases and lysophospholipases results in the formation of GPC [46,51]. Finally, GPC is hydrolysed generating choline (and phosphoglycerol) [16,18,47] or phosphocholine (and glycerol) [48–50]. Another more hypothetical pathway is the 'de-novo' synthesis of GPC, proposing a direct transfer of choline to GPC via CDP choline but not via PC [19–21]. Yet, the experimental evidence for this pathway is discussed quite controversially [18,22–25,78]. In contrast to MDCK cells uptake of GPC from the extracellular compartment seems to be impossible for IMCD cells [16].

Pathways for the synthesis of phosphatidylcholine and glycerophosphorylcholine in IMCD cells

In this study we could confirm the observation that IMCD cells have a potent transport system for choline [16,35]. The maximum velocity of the choline uptake (58 μmol/g cell protein) was close to that found by Bevan and Kinne [28]. Within minutes [*methyl*-^{3}H]-choline was incorporated into a number of cellular components, namely the lipids PC, LPC and SM and

the non-lipids Pcholine, GPC and another yet unidentified water-soluble substance.

To confirm or disprove the abovementioned reaction sequence from choline to GPC via PC for isolated IMCD cells, the transfer of radioactivity in pulse-chase experiments indicated by the change in the GPC/PC ratio was of main interest. The radioactivity initially associated with PC far exceeded that of GPC and other metabolites. In case of GPC synthesis from PC, the radioactivity associated with PC was expected to be transferred to GPC during extended incubations. Therefore, the GPC/PC ratio should be low at the start of the experiment and rise over extended incubation. Indeed in pulse-chase experiments the relative radioactivity of PC expressed as percentage of the total radioactivity decreased significantly within 15 h whereas the relative radioactivity of GPC significantly increased. The GPC/PC ratio of the specific activities also showed a significant increase. GPC was the only metabolite whose specific activity increased significantly with the chase time, indicating that GPC must have been synthesized from radioactive precursors already existing after removal of [*methyl*-^{3}H]choline. Solely PC and LPC had specific activities being high enough to be precursors of GPC. Therefore, it is likely that PC (via LPC) is the direct precursor of GPC. In short-term pulses decreasing ratios of GPC/PC were observed. These could be explained either by a faster labeling of the PC pool presumably via the Kennedy pathway or by the assumption that GPC is a precursor of PC. However, de-novo synthesis of GPC from choline seems to be unlikely, since no radioactive CDP choline – an intermediate of this pathway – could be detected. In case of PC synthesis from GPC the intermediate LPC (termed acyl-GPC by Infante [19]) should be initially labeled faster and to a markedly higher extent than PC especially considering its small pool size (1–2% of PC). This is corroborated by the observation of Limas and Limas [51] that the velocity of lysophospholipase is far exceeding the velocity of the reacylating enzyme acyl-CoA : lysophosphatidylcholine acyltransferase. Therefore, radioactivity would be expected to accumulate in LPC if GPC would be reacylated to PC with the acylation of LPC being slow. We found the initial specific activity of LPC to be significantly lower compared to that of PC pointing to the reverse order, namely, PC degradation via LPC to GPC.

Our results only partly agree with the data obtained by Zablocki et al. [52]. When MDCK cells are grown at 315 mosmol/l in pulse experiments using radioactive choline the specific activity of GPC after 1 h is much higher than that of PC. Grown at 715 mosmol/l – conditions more comparable to our experiments – the maximum ratio of specific activity of GPC/PC is 1 : 1.5 compared to 1 : 11.3 in our experiments. Their results may indicate that appreciable de-novo synthesis of GPC from choline occurs at low osmolarity, whereas in hyperosmotic medium the catabolic synthesis of GPC from PC might be higher. Further studies are neccessary to investigate this possibility.

Additional support for the assumption that GPC is synthesized from PC via LPC is provided when considering the reaction sequence from choline via PC and GPC back to choline in the presence of an inhibitor of PC hydrolysis. Phospholipase A_2 inhibition causes a (partial) blockade of the PC hydrolysis to GPC leading to a significant reduction of the transfer of radioactivity from PC into GPC. The significantly stronger decrease in GPC level than in PC level is consistent with the notion that PC is the GPC precursor. This is confirmed by the significant increase in the specific activity of PC and the lack of a significant change for GPC. The decrease in the amount of PC may be due to a general inhibition of the turnover from choline to GPC supported by a significant increase in the choline level. Due to the length of the pulse period and the limited specificity of quinacrine the data, however, are no proof for GPC synthesis from PC if regarded separately from the other results.

Interestingly, no labeled betaine could be identified in any experiment although several investigators have found betaine and/or betaine synthesis in the kidney, as well as in whole inner medulla [53–56]. However, the synthesis seems to be restricted to the mitochondrial fraction [57–59]. As the content of mitochondria in our pure IMCD cell preparation is quite low [28,60], the lack of betaine labeling might be explained by the fact that other investigators used mitochondria rich cells [55]. However, it might also represent a cell and/or species-type-specific function [61]. The unidentified water-soluble substance may be located at an early step of the sequence because of its fast appearance and disappearance. The chromatographic pattern is similar to that of acetylcholine. As PC, choline and acetylcholine are closely-linked in brain metabolism (for an overview, see Ref. 62), a high transient incorporation of radioactivity in acetylcholine would not be unlikely. An in-vivo incorporation of radioactive choline in acetylcholine has also been found in the kidney of guinea pigs [63]. However, from the pattern in various chromatographic systems we can exclude that the metabolite is identical with acetylcholine, as well as PAF (platelet-aggregating factor), CDP choline, betaine or sarcosine (not shown). Yet, no other laboratory seems to have found or identified this metabolite.

Another peculiarity in our studies is the high radioactive labeling of Pcholine after 15 h. Responsible for that could be a product inhibition of phosphocholine cytidylyltransferase by PC or even GPC. Supposing a low activity, respectively, a low turnover rate under hyperosmotic conditions [52] the findings of several authors that PC inhibits phosphocholine cytidylyl-

transferase may explain the accumulation of radioactive Pcholine [64–66]. Other possibilities for Pcholine synthesis might be the degradation of GPC to Pcholine and glycerol by the action of a phosphocholine diesterase (probably restricted to the brain) [48,49] or phospholipase C, reported in the kidney medulla by Craven and Derubertis [67], which is directly generating Pcholine from PC. However, this seems to be unlikely from our results of the phosphatidyl[*methyl*-^{3}H]choline PC experiments: Initially, very little radioactivity was associated with Pcholine, argueing against involvement of phosphocholine diesterase or phospholipase C.

The rate-limiting step of the reaction sequence from choline to PC seems to be the conversion of Pcholine and CTP to CDP choline. Phosphocholine cytidylyltransferase responsible for this step is known from other investigators to have a low turnover rate [44,45,68]. Newly-formed CDP choline is therefore rapidly converted to PC. This is in accordance with our own results where radioactively-labeled CDP choline could not be found under any conditions, although in other laboratories (e.g., Ref. 18) CDP choline could be identified as a minor intermediate ($< 0.5\%$ of total radioactivity).

Pathways for phosphatidylcholine degradation

The results obtained using radioactive PC were similar to those using radioactive choline but allowed to focus on the PC degradation. In pulse-chase experiments the specific activity of PC decreased significantly from 1 h to 15 h. Correspondingly, the specific activities of LPC, GPC and choline increased significantly with the increase for GPC being highest. From these results it may be concluded that GPC is directly synthesized from PC (via LPC) as stated before, since no other radioactive metabolite, except choline (minor labeling of Pcholine, see above), could be found. The stronger labeling of choline compared to GPC, especially at short time intervals, may be due to the direct action of membrane-bound phospholipase D on PC [69]. Phosphatidyl[*methyl*-^{3}H]choline is probably first entering the outer leaflet of the membraneous compartment. As an exchange of lipid molecules from the outer to the inner leaflet is a rare process [70], the exchange with the metabolically active PC pool will be slow and therefore cause a retention of radioactivity. This may cause an extended action of phospholipase D on PC which is normally not accessible to the enzyme. In the cytosolic compartment mainly phospholipase A_2 (and phospholipase C) acts on PC [71,72]. The possibility of heterogeneous PC pools was proposed by several authors [52,73–75]. Therefore, the specific activity of GPC compared to choline is much higher when radioactive choline is used instead of radioactive PC. In addition, the specific activity of GPC released into the incubation medium by the cells is approx. 15-fold higher using radioactive choline than using radioactive PC (not shown) which means that GPC is synthesized intracellularly from a PC pool different from membraneous PC. Other investigators provided evidence that the reaction sequence from choline to PC is 'channeled', which means that the enzymatic reactions are closely coupled and metabolites are not freely diffusible within the cell [24]. This would also explain different GPC/PC ratios in specific activity obtained using radioactive PC or after labeling of PC with radioactive choline. In the former case most of the labeled PC, due to its predominant occurrence in the outer leaflet of the plasma membrane [76], is probably not accessible to hydrolysis by phospholipases acting on the inner leaflet of the membrane.

In view of the major pathway of PC hydrolysis our data agree with Morash et al. [18] who found the degradation of PC via LPC to GPC to be the main reaction in a variety of cell types.

Pathways for glycerophosphorylcholine degradation

Several possibilities for the degradation of GPC exist. Enzymatic reactions include hydrolysis of the ester bonds between choline and phosphoglycerol as well as between Pcholine and glycerol. The enzymes responsible for these reactions are GPC:choline diesterase and GPC:phosphocholine diesterase [48–50,77]. In our studies no evidence for the presence of phosphocholine diesterase could be provided. This can be deduced mainly from the phosphatidyl[*methyl*-^{3}H]choline experiments where we failed to detect significant initial Pcholine labeling. Therefore, the initial

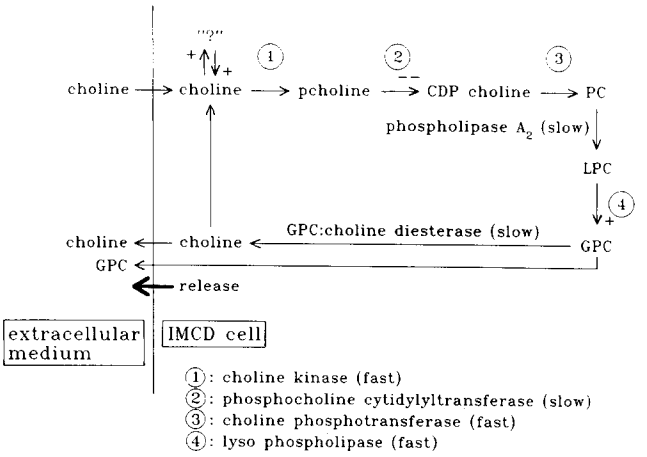

Fig. 3. Reaction sequence for the metabolism of GPC. Choline is taken up from the medium subsequently phosphorylated to Pcholine and linked to CDP choline with CTP. Under release of CMP the activated form is transferred to a diglycerol the reaction product being PC. By a stepwise cleavage of the fatty acid residues, LPC and GPC originate followed by the hydrolysis of GPC to choline (and phosphoglycerol) catalysed by the GPC:choline diesterase. The cellular GPC concentration is (partly) regulated by GPC release.

product of GPC hydrolysis is exclusively choline and not Pcholine. The very slow increase in the Pcholine labeling over an extended incubation period is probably due to the phosphorylation of choline released from GPC and/or PC.

The most probable reaction sequence deduced from our results is shown in Fig. 3. Concerning the velocity of all reactions the terms 'slow' and 'fast' are relative. The reactions of choline kinase, phosphocholine cytidylyltransferase and choline phosphotransferase may be fast in the beginning compared to phospholipase A_2, lysophospholipase and GPC:choline diesterase. However phosphocholine cytydylyltransferase is postulated to have the lowest turnover rate of all involved enzymes. Further work with direct measurements of single enzymes has to be done to make a definitive statement about the rate-limiting reaction(s) of the sequence.

In conclusion it can be stated that IMCD cells possess the complete reaction sequence for the synthesis, as well as for the degradation of the organic osmolyte GPC from its precursor choline. A very important intermediate of the sequence is the membrane lipid PC. Thereby, two pools of PC seem to exist: one used as a GPC precursor pool, the other related to overall plasma membrane integrity. The possibility whether also 'de-novo' synthesis of GPC may occur under certain conditions remains to be elucidated.

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