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Dexamethasone and Cyclic AMP Regulate Sodium Phosphate Cotransporter (NaPi-IIb and Pit-1) mRNA and Phosphate Uptake in Rat Alveolar Type II Epithelial Cells

Chengluo Jin · Evangelos Zoidis · Claudia Ghirlanda · Christoph Schmid

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Abstract Alveolar epithelial type II (AT II) cells need phosphate (Pi) for surfactant synthesis. The Na-dependent (Na_d) Pi transporters NaPi-IIb and Pit-1 are expressed in lung, but their expression, regulation, and function in AT II cells remain unclear. We studied NaPi-IIb and Pit-1 mRNA expression in cultured AT II cells isolated from adult rat lung, their regulation by agents known to enhance surfactant production, dexamethasone (dex) and dibutyryl cyclic AMP (cAMP), and the effects of dex and cAMP on Na_d Pi uptake by this cell type. By Northern analysis, cultured AT II cells expressed both NaPi-IIb (4.8 and 4.0 kb) and Pit-1 (4.3 kb) mRNA. Treatment with 100 nmol/l dex for 24 h decreased the expression of both mRNAs (to 0.48 ± 0.06 and 0.77 ± 0.05, respectively, as compared to control), while 0.1 mmol/l cAMP stimulated NaPi-IIb (1.94 ± 0.22) but not Pit-1 mRNA (0.90 ± 0.05, compared to vehicle-treated cells). NaPi-IIb and Pit-1 proteins could not be identified by western analysis of plasma membrane preparations of cultured AT II cells. AT II cells take up Pi in a Na_d manner. Uptake was slightly (to 0.78-fold of the control) decreased by 100 nmol/l dex but not affected by 0.1 mmol/l cAMP treatment. Although NaPi-IIb mRNA expression was maintained to some extent by AT II cells kept in primary culture, Pi uptake was more closely related to Pit-1 mRNA expression.

Keywords Dexamethasone · Cyclic AMP · Sodium-dependent phosphate transport · Phosphate transporter Pit-1-NaPi-IIb mRNA · AT II cells

C. Jin · E. Zoidis · C. Ghirlanda · C. Schmid
Division of Endocrinology and Diabetology, Department of Internal Medicine, University Hospital, 8091 Zurich, Switzerland

Present Address:
C. Jin
Department of Surgery, Second Affiliated Hospital, Harbin Medical University, Harbin, China

Present Address:
E. Zoidis (✉)
Faculty of Animal Science and Aquaculture, Department of Nutritional Physiology and Feeding, Agricultural University of Athens, 118 55 Athens, Greece
e-mail: ezoidis@aua.gr

Introduction

Apart from serving as a progenitor for the type I pneumocyte (AT I) cell, the main role of the alveolar epithelial type II (AT II) cell is pulmonary surfactant (PS) synthesis and secretion. PS decreases surface tension at the air-liquid interface and prevents alveolar collapse at the end of expiration. Isolated PS consists of approximately 90% lipid, 10% protein, and traces of carbohydrate [1]. The lipids are largely phospholipids (phosphatidylcholine), with some triacylglycerols and cholesterol. PS contains four unique, lung-specific proteins: surfactant protein (SP)-A, -B, -C, and -D. Surfactant phospholipids are stored in lamellar bodies, the secretory granules in the AT II cells, and secreted by exocytosis [2, 3]. The majority of secreted PS is removed from the alveolar space by reuptake into AT II cells. As an essential element in PS, phosphate (Pi) is taken into AT II cells.

Several sodium-coupled Pi transport proteins (NaPi) have been identified, which enable intracellular uptake of Pi by taking advantage of the steep extracellular-to-intracellular sodium gradient, and have been classified into three groups (types I, II, and III) [4, 5]. Among them, NaPi-II (including subtypes NaPi-IIa and NaPi-IIb) plays a key

role in Pi homeostasis. NaPi-IIa is expressed in apical membranes of epithelial cells in renal proximal tubules and represents the major NaPi cotransporter in the kidney [6, 7]. NaPi-IIa is regulated by dietary Pi, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, and glucocorticoids [6]. NaPi-IIb (*SLC34A2*) is expressed in several tissues, including the brush border membranes of the small intestinal epithelium [8], where it is thought to be the major NaPi cotransporter. Intestinal NaPi-IIb is regulated by dietary Pi, glucocorticoids, and 1,25-dihydroxyvitamin D_3 [9–12]. NaPi-IIb is also expressed in AT II cells in lung [13, 14]. Dietary Pi does not appear to be a regulatory factor of NaPi-IIb expression in AT II cells [13], indicating distinct regulation of NaPi-IIb in different tissues. Mutations in the *SLC34A2* gene and functional NaPi-IIb deficiency have been found to be associated with and to cause pulmonary alveolar microlithiasis (PAM), an autosomal recessive disorder in which microliths are formed in the alveolar space [15, 16].

NaPi-III are ubiquitously distributed NaPi cotransporters that have been separated in two distinct subtypes, Pit-1 and Pit-2 [17, 18]. Pit-1 and Pit-2 belong to the *SLC20* family of solute carriers [19]. Pit-1 mRNA expression is upregulated in vitro by Pi deprivation [20, 21], and it has been suggested that Pit-1 and related proteins (induced by Pi starvation) may act as primary Pi-sensing proteins [21, 22]. Pit-1 mRNA expression is particularly abundant not only in brain and bone but also in tissues rich in those polarized epithelial cells (intestine and lungs), which also express NaPi-IIb [21–24]. Although NaPi-IIb and Pit-1 are expressed in lung, their function and regulation in lung alveolar cells remain unknown.

Glucocorticoids and cAMP play an important regulatory role in AT II cells during fetal lung maturation and AT II cell character maintenance. A number of genes are regulated by glucocorticoids, including those encoding the SPs, lipogenic enzymes, water and ion transporter/channels, and others. Glucocorticoids and cAMP have synergistic stimulatory effects on SPs mRNA expression in cultured AT II cells [25–28]. Dex and cAMP also upregulate Na^+ transport in alveolar epithelial cells by stimulating the α subunit of the epithelial Na^+ channel (ENaC) and Na^+-K^+-ATPase gene expression [29]. Active transepithelial transport of sodium is important since fluid is thereby absorbed from the alveoli to the interstitium.

Pi uptake by AT II cells has been reported to be Na_d and to decrease in parallel with surfactant synthesis over time in primary culture [30], but the NaPis involved and their regulation by hormones and growth factors have not been studied.

In this study, we investigated NaPi-IIb and Pit-1 mRNA expression in AT II cells isolated from adult rat lung and kept in primary culture for 2 days, and their regulation by dex and/or cAMP. We also studied the effects of dex and cAMP on Na_dPi uptake in this cell type, and we tested whether the effects of these agents were similar in the rat lung cell line L2 that is derived from spontaneously transformed AT II cells [31].

Materials and Methods

Cell Isolation

AT II cells were isolated from adult male Wistar rats (150 g, Harlan, The Netherlands) by a procedure originally described by Dobbs et al. [32] with modifications of the methods described by Murphy et al. [33]. Usually four animals were used and the isolated cells combined for an experiment. All animal experiments were approved by the institutional Animal Welfare Committee. Animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight, Abbott Laboratories, Abbott Park, IL, USA). The abdomen was opened and sodium heparin (2000 I.E./kg body weight, Brown, Emmenbrücke, Switzerland) was given via the portal vein. After cannulating the trachea, the blood cells were removed from the lung by perfusing the pulmonary artery with saline (37°C), while the lung was ventilated in situ with 8-10 ml of air by means of a syringe attached to the cannula. The blood-free lung (appearing completely white) was removed from the thoracic cavity with the trachea and lavaged six times with 10 ml of saline (37°C). The lung lavages were pooled per animal and centrifuged to collect alveolar macrophages. Collected rat lung macrophages were cultured for further studies. The airways of the lavaged lung were digested by trypsin/elastase solution (trypsin: 0.5%, Invitrogen, Carlsbad, CA, USA; elastase: 3 U/ml, Worthington, NJ, USA; diluted with Dulbeco's buffer, pH 8.0) administered through a cannula inserted into the trachea for 40-50 min at 37°C. The digestion was stopped by 5 ml of fetal calf serum (FCS, Invitrogen) and the isolated cell suspension was filtered through gauzes, 140- and 20-μm nylon mesh (Millipore, Bedford, MA, USA) sequentially. Cells were purified by incubation in bacteriologic dishes (10-cm diameter, Greiner Bio-One GmbH, Frickenhausen, Germany) covered with rat IgG (Sigma, St. Louis, MO, USA) for 1 h at 37°C, 5% CO_2:air. The unattached cells were collected and cultured for further studies.

Culture Conditions

Isolated AT II cells were cultured on type I collagen (Collagen S, Roche Diagnostics GmbH, Mannheim, Germany)-precoated Falcon dishes (Becton–Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 4×10^5 cells/cm^2

in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 μg/ml), glutamine (2 mmol/l), and 10% fetal calf serum (FCS, Invitrogen). After 20-24 h of culture, the medium was removed and fresh DMEM/Ham's F12 medium (1:1 mixture) containing 1 g/l bovine serum albumin (BSA, Invitrogen) was added with/without test compounds, dex (Sigma) and/or cAMP (Sigma) for an additional 24 h for RNA extraction or 22 h for Pi uptake studies. cAMP was directly dissolved in BSA-containing test culture medium, dex (stock, 10^{-3} mol/l) in absolute ethanol (and controls adjusted to the final concentration of the vehicle, 0.01% for 100 nmol/l dex). Recombinant human insulin-like growth factor I (IGF-I) was from Ciba-Geigy (Basel, Switzerland), human keratinocyte growth factor (KGF, formerly FGF-7) from Sigma, and vascular endothelial growth factor (VEGF) and transforming growth factor (TGF-β_2) from R&D Systems (Abingdon, UK).

L2 Cells

L2 cells, a rat lung cell line derived from type II alveolar epithelial cells [31], were passaged in Falcon tissue culture flasks in DMEM supplemented with gentamicin (50 μg/ml), glutamine (2 mmol/l), and FCS (5%). Cultures between passages 12 and 28 were used. Cells grown to confluence were detached from the flasks with 0.25% trypsin and replated in multiwell tissue culture plates (Falcon, 35-mm diameter) at a density of 2×10^5 cells per well in DMEM containing 5% FCS. Confluent monolayers formed 3 days after seeding. Cell cultures were rinsed with serum-free medium and kept in Ham's F12 medium containing gentamicin, glutamine, and BSA (1 g/l) for the last 24 h. Aliquots of test agents were added directly to the media as indicated.

Total RNA Isolation

The culture medium was sucked off and the cell layer was washed three times with ice-cold phosphate-buffered saline (PBS) and scraped into ice-cold PBS. Cells were collected and then lysed in 4 M guanidine isothiocyanate containing 5 mmol/l sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, and 0.5% sarcosine. Total RNA was obtained by high-speed sedimentation through a cesium chloride cushion, and concentrations were determined spectrophotometrically (1 $OD_{260nm} = 40$ μg/ml RNA); RNA was stored at -80°C until assayed [34].

Northern Blot Analysis

Denatured total RNA (20 μg) from tissues and cells was electrophoresed on a 1% agarose gel (stained with ethidium bromide) containing 2 M formaldehyde, transferred onto a nylon membrane (Hybond-XL, Amersham, UK) by capillary blotting, and fixed by UV crosslinking according to standard procedures [35]. Prehybridization and hybridization were performed as described earlier [21]. The following cDNAs [made by reverse transcription (RT)-PCR in our laboratory using primers listed in Table 1] were used for hybridization: rat SP-B (nucleotide 693-1044, GenBank Accession No. X14778), rat SP-C (nucleotide 122-455, GenBank Accession No. NM017342), rat NaPi-IIb (nucleotide 624-1421, GenBank Accession No. AF 157026) [14], and rat Pit-1 (nucleotide 133-1431, GenBank Accession No. AB000489) [22]. The cDNA probes were labeled by random primer extension using a commercial kit (Roche Diagnostics, Indianapolis, IN, USA) and [α-^{32}P] deoxy-CTP (3000 Ci/mmol; Amersham) to specific activities of $2\text{-}4 \times 10^9$ cpm/μg DNA. After 14-16 h of incubation at 42°C, the membranes were washed twice for 15 min at room temperature in 2× SSPE—0.1% SDS and then at 49-52°C twice for 10 min in 0.1× SSPE—0.1% SDS. Membranes were then exposed at -80°C to a BioMax film (Kodak, Rochester, NY, USA) in cassettes equipped with intensifying screens to visualize ^{32}P-labeled cDNA-mRNA hybrids. mRNA levels were quantitated by scanning densitometry using a Bio-Rad (Hercules, CA, USA)

Table 1 Oligonucleotide primers used in this study

Name	Strand	Primer sequence	Position[a]	Species[b]	GenBank accession no.
SP-B	Sense	CCTGGTGGTGGGTGGCATCT	693	Rat	X14778
SP-B	Antisense	TGTGGGCATCCTGGCTCCTA	1044	Rat	X14778
SP-C	Sense	TCATCGTGGTTGTTGTGGTA	122	Rat	NM017342
SP-C	Antisense	AGAGGTGGGTGTGGAGGACT	455	Rat	NM017342
NaPiIIb	Sense	TGTCCATGACTTCTTCAACT	624	Rat	AF157026
NaPiIIb	Antisense	AATGGCAGTCGTGGTGGTGC	1421	Rat	AF157026
Pit1	Sense	TGTTACTGCTTCTGCTCCAC	133	Rat	AB000489
Pit1	Antisense	ATTTCGTCTCCCTTTTGTTC	1431	Rat	AB000489

[a] Position refers to the nucleotide position within the respective sequence where the 5′ end of the primer will anneal

[b] Species from which the primers were designed

video densitometer. Variations of gel loading were corrected against 18S ribosomal RNA levels.

Pi Transport Studies

Before the start of experiments, monolayers of AT II cells were washed with 1 ml transport buffer A or B containing (in mmol/l): 140 NaCl or choline chloride, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 15 *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), adjusted to pH 7.4 with Tris–(hydroxy-methyl) aminomethane HCl. Transport buffer B containing choline chloride instead of NaCl was used for measuring Na^+-independent Pi uptake. The ^{32}P uptake studies were performed at room temperature and initiated by adding 1 ml transport buffer containing 0.1 mmol/l $KH_2{}^{32}PO_4$ (1 μCi/ml, 200 mCi/mmol; Amersham). Ten minutes later, the buffer was removed and the dishes were quickly rinsed three times with 1 ml ice-cold transport buffer. The cells were then solubilized with 1 ml of 2% SDS. The radioactivity of a 0.5-ml aliquot was counted in a liquid scintillation counter (Betamatic, Kontron Instruments AG, Zurich, Switzerland). Na_dPi transport was calculated by subtraction of the Na-independent component from the total Pi uptake in the presence of Na and expressed in nmol/mg protein × 10 min; protein content was determined from parallel dishes by the bicinchoninic acid (BCA) method [21, 36].

Western Immunoblot Analysis for NaPi-IIb and Pit-1

Protein was extracted from cell membranes (tissues and cultured cells) and subjected to SDS-PAGE (7.5%), then transferred to either a 0.2-μm nitrocellulose membrane (Bio-Rad 162-0147) or a 0.45-μm PVDF membrane (Hybond P, Amersham RPN303F) for western analysis. Equal loading was confirmed by Ponceau S staining. Filters were incubated overnight at 4°C in primary antibody [all rabbit polyclonal, anti NaPi-IIb (NPT2b 11-S) and anti-Pit-1 (PiT/GLVR-1 11-S) from Alpha Diagnostic International, San Antonio, TX, USA, at 1:1000] and were then washed and incubated for 1 h in secondary antibody (goat anti-rabbit HRP, BioRad 170-6515, at 1:3000) at room temperature. After washing, proteins were detected by using the enhanced chemiluminescence (ECL) detection reagents (Amersham 1059250) applied as recommended by the manufacturer (Amersham) and by exposure to light-sensitive film (Kodak 8194540).

Tissue Homogenization, Protein Extraction, Cell Fractionation

Tissue pieces of 120-g rats were put into 0.5% Triton-X-100, 50 mmol/l HEPES (pH 7.5), 140 mmol/l NaCl, 1 mmol/l PMSF, 3 μg/ml aprotinin, 3 μg/ml leupeptin; mixed to homogeneity; and centrifuged at 16,000*g* at 4°C for 10 min. The supernatant was transferred to an Eppendorf tube and frozen at -80°C.

To improve sensitivity and specificity for NaPi-IIb and Pit-1 detection by western, we used lysis buffer as described, followed by a sequential differential centrifugation as described by Clark et al. [37] with minor modifications in order to obtain the plasma membrane (PM) fraction [38, 39].

Statistical Analysis

Results are expressed as mean ± SEM, as indicated, and are representative of at least three independent experiments. Statistical analysis was performed by ANOVA and Dunnett's post hoc test, with $p < 0.05$ considered statistically significant.

Results

AT II Cell Isolation and Characterization

AT II cells were isolated from adult rats by methods originally described by Dobbs et al. [32] and modified by Murphy et al. [33]. For most experiments, cells were prepared from four animals, typically yielding between 5 and 7.5 million cells per rat. After 48 h of culture on type I rat collagen-precoated plates (cells were washed and medium was changed after 24-h culture), AT II cells attached and

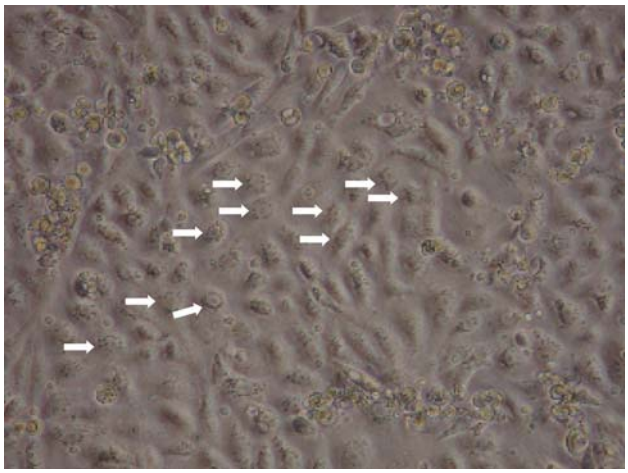

Fig. 1 Forty-eight-hour culture of rat AT II cells. After purification, isolated cells were cultured on type I collagen-precoated Falcon dishes (see "Materials and Methods" section) at a density of 4×10^5 cells/cm^2 and incubated in humidified air/5% CO_2 at 37°C. After 20–24 h of culture, the medium was removed and replaced with fresh serum-free (BSA-containing) medium for additional 22–24 h. Cells with lamellar bodies can be considered to be AT II cells (as indicated by *white arrows*)

showed cuboid-like morphology as shown in Fig. 1. Cells containing lamellar bodies can be considered to present AT II cells because surfactant-rich lamellar bodies are special organelles in AT II cells [40]. Since SP-B and SP-C are specific markers of AT II cells, we checked our AT II cell isolation and culture conditions by studying the expression of these genes. As shown in Fig. 2, both SP-B and SP-C mRNA were expressed in lung tissue (lane 1) but were not detectable in lung macrophages (lane 2). Cells purified (lane 4) by the panning method on rat IgG-precoated plates (see "Materials and Methods" section) were relatively enriched in both SP-B and SP-C mRNA compared to cells before IgG panning (lane 3). Both mRNA levels were much lower in cultured than in freshly isolated cells and decreased further until 48 h (lanes 5 and 6). Loss of SP-C mRNA was even more dramatic than loss of SP-B mRNA within the 2 days the isolated cells were kept in vitro.

In contrast to AT II cells in primary culture, L2 cells could be plated on plastic dishes and grew rapidly.

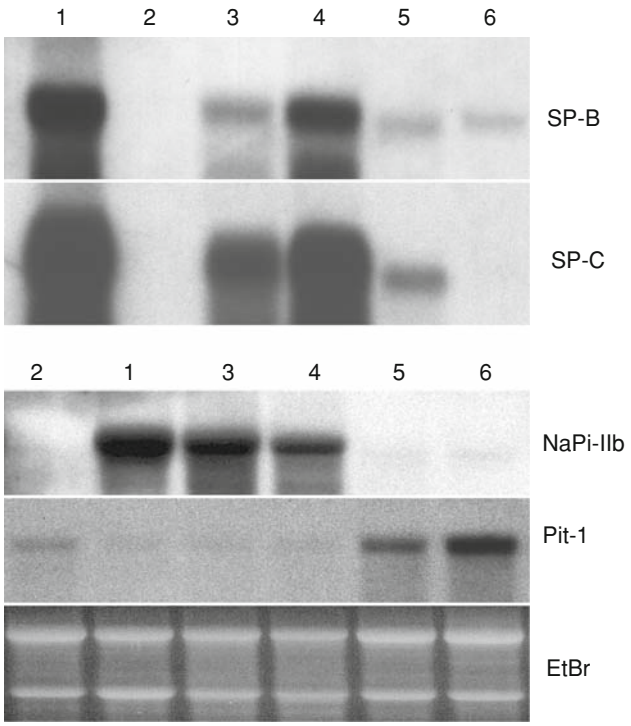

Fig. 2 SP-B and SP-C (*upper panels*) and NaPi-IIb and Pit-1 (*lower panels*) mRNA expression during different steps of cell isolation and after 24 and 48 h of cell culture. Twenty micrograms of the total RNA isolated from blood-free (after lavage) lung tissue (1), lung macrophages (2), cells before rIgG panning (3), cells after rIgG panning (4), and cells cultured for 24 h (5) and 48 h (6). RNA was separated on a 1% agarose formaldehyde gel, blotted on a nylon membrane, and hybridized with ^{32}P-labeled rat SP-B, SP-C, NaPi-IIb, Pit-1, and 18S cDNA probes. Signals were visualized by autoradiography (see "Materials and Methods" section). Ethidium bromide staining of 28S and 18S ribosomal RNA bands is also shown to illustrate equal loading. One representative blot of mRNA expression for all treatments (with comparable outcomes) is shown

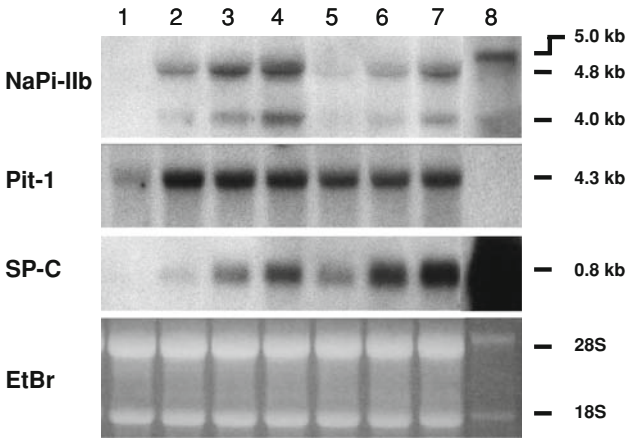

Fig. 3 NaPi-IIb, Pit-1, and SP-C mRNA expression in cultured L2 and AT II cells and in rat lung. Twenty micrograms of total RNA extracted from cultured L2 (*lane 1*) and AT II cells (*lanes 2–7*) treated for 24 h with vehicle (2), 8-BrcAMP 0.1 mmol/l (3), dbcAMP 0.1 mmol/l (4), dex 100 nmol/l (5), dex 100 nmol/l + 8-BrcAMP 0.1 mmol/l (6), or dex 100 nmol/l + dbcAMP 0.1 mmol/l (7), and 5 μg from adult rat lung (8) were separated on a 1% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled rat NaPi-IIb, Pit-1, SP-C, and 18S cDNA probes as described in "Materials and Methods" section. Signals were detected by autoradiography. The *lower panel* represents ethidium bromide staining of 28S and 18S ribosomal RNA bands to indicate equal loading (20 μg in the case of RNA from cultured cells). One representative blot

However, SP-B and SP-C mRNA could not be detected by northern analysis in L2 cells (not shown, Fig. 3).

Northern Blot Analysis for NaPi-IIb and Pit-1 mRNA

NaPi-IIb and Pit-1 mRNA are detected in adult rat lung; subsequent to isolation and growth in culture, NaPi-IIb mRNA is decreased while Pit-1 mRNA is markedly increased (Fig. 2). To determine the effect of dex and cAMP on NaPi-IIb and Pit-1 mRNA expression, AT II cells were treated for 24 h with vehicle, 100 nmol/l dex, 0.1 mmol/l cAMP, or a combination of them. We also tested SP-C mRNA regulation by these two agents for comparison and as control. Northern blots hybridized with NaPi-IIb cDNA probe revealed two bands (~4.8 and 4.0 kb) in cultured AT II cells, and a prominent band (~5.0 kb) in lung tissue (Figs. 2, 3). Pit-1 mRNA was visualized by Northern as a 4.3-kb band, and the transcript size was the same in RNA from lung, cultured AT II cells, and L2 cells. First, we tested 8-Br cAMP and dibutyryl cAMP (both at 0.1 mmol/l) in AT II cells. Since dibutyryl cAMP was more potent in stimulating NaPi-IIb and SP-C mRNA expression than 8-Br cAMP (lanes 2 and 3), dibutyryl cAMP was used for all later experiments. Densitometric quantitation of Northern blot films showed that dex significantly decreased NaPi-IIb and Pit 1 mRNA expression [to 0.48 ± 0.06 and 0.77 ± 0.05, respectively, of the control (Fig. 4a, b), $p < 0.01$], but

slightly increased SP-C mRNA [1.86 ± 0.22 (Fig. 4c)]. cAMP increased NaPi-IIb mRNA (1.94 ± 0.22, $p < 0.01$), had no effect on Pit-1 mRNA (0.90 ± 0.05), and increased SP-C mRNA (2.52 ± 0.50) (Fig. 4a–c). Combined treatment (dex and cAMP) tended to increase NaPi-IIb mRNA (1.29 ± 0.05), decreased Pit-1 mRNA (0.66 ± 0.04, $p < 0.01$), and increased SP-C mRNA (4.71 ± 0.84, $p < 0.01$, $n = 4$) expression (Fig. 4a–c).

Pit-1 but not NaPi-IIb mRNA expression could be detected in L2 cells; however, the 4.3-kb transcript was a lower-intensity signal in L2 than in AT II cells (Fig. 3). Pit-1 mRNA was decreased in L2 cells exposed to 100 nmol/l dex for 24 h but not affected by cAMP.

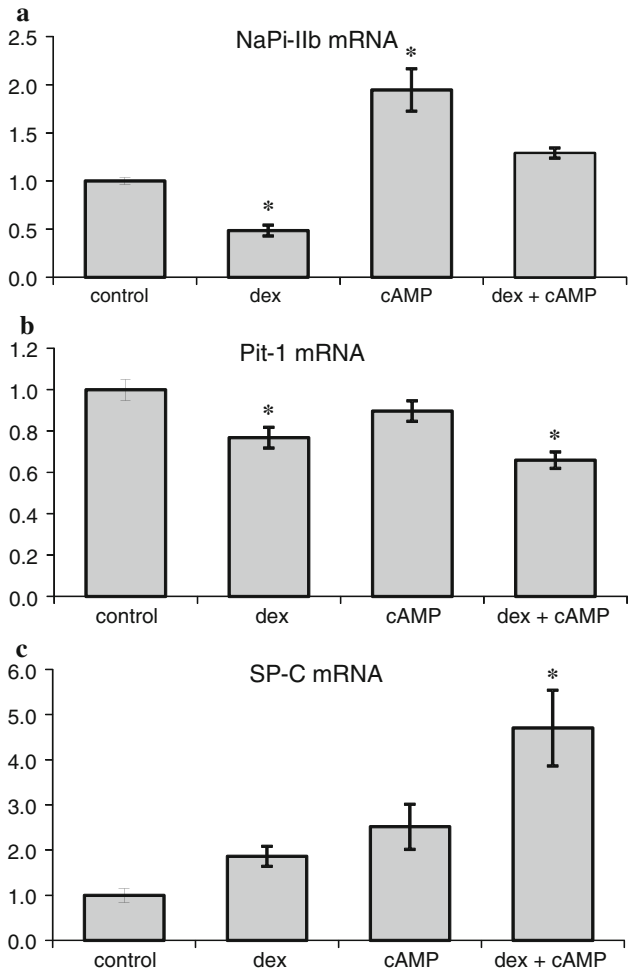

Fig. 4 Pooled densitometry data of northern blots of experiments testing the effects of dex and cAMP on NaPi-IIb, Pit-1, and SP-C mRNA expression in cultured AT II cells. Twenty micrograms of total RNA from cultured AT II cells treated for 24 h with vehicle, 100 nmol/l dex, 0.1 mmol/l cAMP, or a combination of 100 nmol/l dex and 0.1 mmol/l cAMP were separated on a 1% agarose formaldehyde gel, blotted on a nylon membrane, and hybridized with ^{32}P-labeled probe as in Fig. 3 and described in "Materials and Methods" section. NaPi-IIb (**a**), Pit-1 (**b**), and SP-C (**c**). Signals were detected by autoradiography, normalized for equal loading (18S rRNA values), and quantitated by scanning densitometry. Data are shown as treated compared to control (mean ± SEM, $n = 4$ separate experiments). * $p < 0.01$ for the comparison of treatment versus control

Characteristics of Pi Uptake and Effects of Dex and cAMP on Na_dPi Uptake

To study the influence of dex and cAMP treatment on Pi transport in cultured AT II cells, we measured Pi uptake in cultured AT II cells treated with or without 100 nmol/l dex and 0.1 mmol/l cAMP for 22 h. First, we measured Pi uptake over different incubation times and in the presence of different Pi concentrations, in Na-containing buffer and in Na-free buffer. Pi uptake (during 10 min of incubation) as a function of extracellular Pi concentration is shown in Fig. 5. Uptake of Pi was lower when Na was replaced by choline, i.e., in the absence of Na. The kinetic parameters of the Na_dPi transport system were obtained by Lineweaver-Burk plot analysis from such experiments where Pi transport was studied over a range of Pi concentrations from 0.02 to 2 mmol/l. Data from four independent curves were combined to estimate a mean apparent affinity constant (K_M) for Pi of 46 ± 7 μmol/l. Subsequent uptake studies were carried out in the presence of 0.1 mmol/l Pi, i.e., at a Pi concentration slightly above the K_M but well below physiological extracellular Pi levels (∼1 mmol/l in human and ∼2 mmol/l in rat plasma). After 10 min of incubation at 0.1 mmol/l Pi, the Na-independent Pi uptake amounted to less than 8% of the total Pi uptake (Fig. 5). Na_dPi uptake in AT II cells was also measured over different time periods after cells had been treated for 22 h with/without 100 nmol/l dex (Fig. 5) or 0.1 mmol/l cAMP (not shown). Na_dPi uptake by vehicle-, dex-, and cAMP-treated AT II cells increased linearly with time (5, 10, and 20 min).

Na_dPi uptake was lower in cells treated with 100 nmol/l dex for 22 h than in control AT II cells (Fig. 5, Table 2), while 6 and 2-h treatment had no such effect (Fig. 6). Treatment with 0.1 mmol/l cAMP had no effect on Na_dPi uptake after 2, 6, and 22 h (Fig. 6). Na_dPi uptake was slightly but consistently decreased by 22-h 100-nmol/l dex treatment, not affected by 0.1 mmol/l cAMP, and slightly decreased by 100-nmol/l dex plus 0.1-mmol/l cAMP treatment (Table 2). Treatment with 1 nmol/l IGF-I for 22 h increased Na_dPi uptake (to 1.32 ± 0.08-fold of the control, three experiments in triplicate), while 10 ng/ml KGF and 100 ng/ml VEGF had no effect (not shown); 0.1 nmol/l TGF-β tended to increase (to 1.17 ± 0.10 of control) Na_dPi uptake.

Na_dPi transport as expressed in nmol/mg protein × 10 min was higher in AT II cells than in L2 cells (Table 2). In the latter, both dex and cAMP treatment decreased Na_dPi transport (Table 2). Treatment with

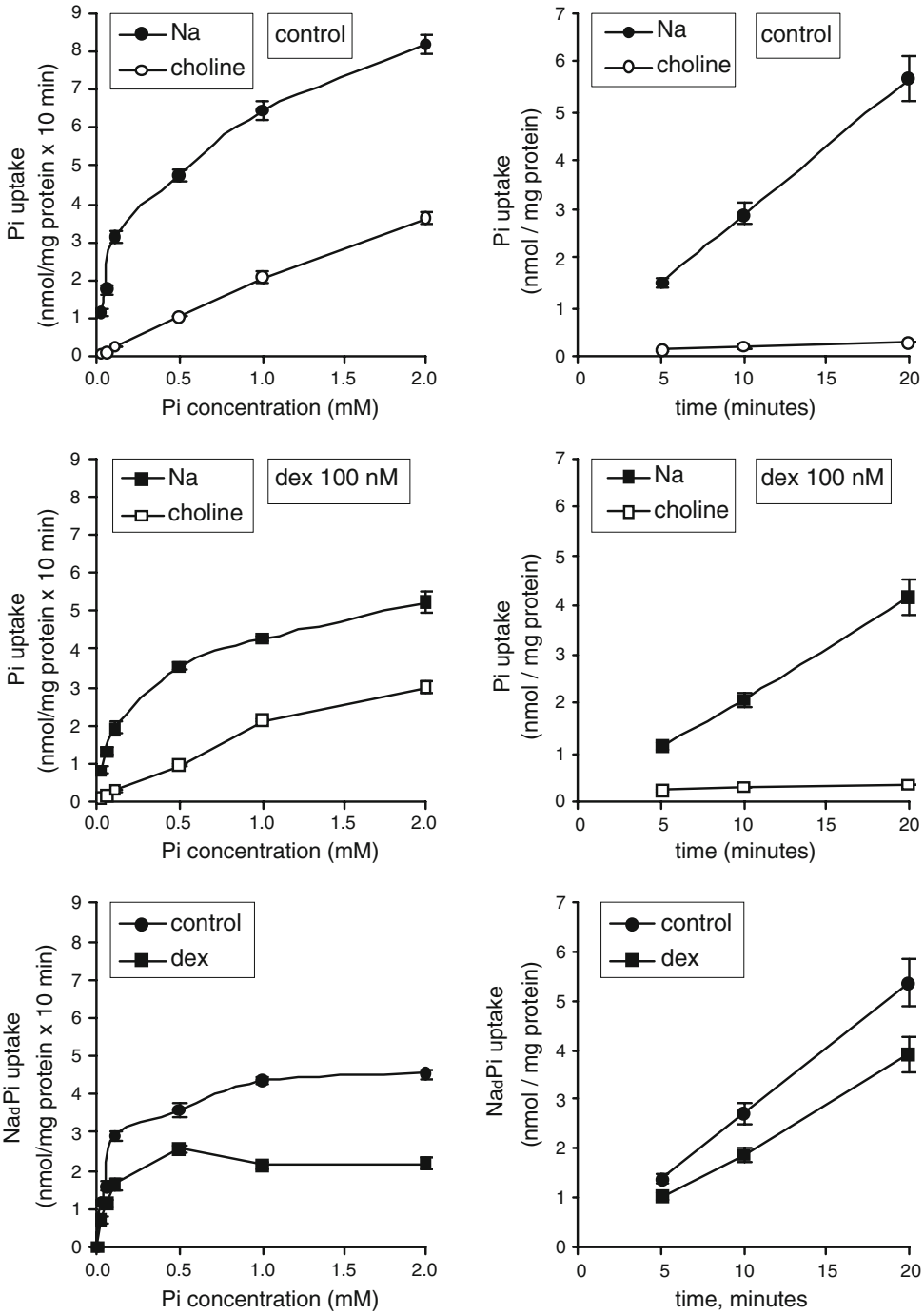

Fig. 5 Effects of Pi concentration (*left*) and of different incubation times (*right panels*) and of dex pretreatment on Pi uptake assessed in Na-containing and in Na-free buffer. AT II cells were cultured in 10% FCS DMEM for 22 h, then in 1 g/l BSA F12 serum-free medium for 22 h, supplemented with dex or vehicle. Pi uptake was measured over 10 min (*left*) and at a Pi of 0.1 mmol/l (*right*) in the presence of Na or choline for 5, 10, and 20 min. Na_dPi uptake was calculated by subtracting the uptake in choline-containing buffer from the uptake in Na-containing buffer and expressed per mg protein as described in "Materials and Methods" section. Results represent mean ± SEM of two separate experiments performed in triplicate

1 nmol/l IGF-I for 22 h increased Na_dPi uptake (to 1.45 ± 0.14-fold of the control, three experiments in triplicate).

Western Blot Analysis for NaPi-IIb and Pit-1

NaPi-IIb and Pit-1 proteins could not be detected in plasma membranes prepared from cultured AT II cells using the antibodies available to us.

Discussion

In this study we investigated NaPi-IIb and Pit-1 gene expression as well as Pi uptake in cultured rat AT II cells. Under a light microscope, cultured cells showed AT II cell-characteristic phenotype. According to northern blot analysis, mRNA for SP-B and SP-C, two AT II cell-specific markers, were expressed in lung tissue and during the different steps of isolation. Higher relative SP-B and SP-C

Table 2 Na_dPi uptake in cultured AT II and L2 cells

Treatment	Na_dPi uptake (nmol/mg protein × 10 min)	
	AT II cells	L2 cells
Control	2.37 ± 0.20	1.27 ± 0.07
cAMP	2.89 ± 0.28	1.11 ± 0.07
Dex	1.89 ± 0.16*	0.75 ± 0.08*
Dex + cAMP	2.39 ± 0.23	0.61 ± 0.09*

Effect of dex and cAMP on Na_dPi uptake in cultured AT II and L2 cells. Rat AT II cells were cultured in 10% FCS DMEM for 22 h, L2 cells for 3 days in 5% FCS-containing medium, then both cell types were treated in 1 g/L BSA F12 serum-free medium with/without dex 100 nmol/l and/or cAMP 0.1 mmol/l for 22 h. Na_dPi uptake was determined as described in Fig. 5 and in "Materials and Methods" section. Data are mean ± SEM of four separate experiments performed in triplicate

* $p < 0.05$ for the comparison of dex versus control

mRNA abundance was observed after purification, but the initially high levels of expression rapidly decreased with time in culture, characteristics which all fit to isolated AT II cells and previous reports [27, 41, 42]. Maintenance of AT II cell-specific features was suggested by SP-B and SP-C mRNA regulation in our isolated cells. SP-B mRNA level in cultured AT II cells was stimulated by 10 ng/ml KGF but not that of SP-C (data not shown) as reported by others [43, 44]. However, 100 ng/ml VEGF and 1 nmol/l IGF-I did not affect SP-B and SP-C mRNA. VEGF has more recently been reported to increase SP production, whereas IGF-I was found to stimulate ^{3}H-choline incorporation into phosphatidylcholine of PS and residual fractions several years ago [45–48]. SP-C mRNA expression was consistently enhanced by dex and cAMP treatment, in agreement with repeatedly confirmed observations [26–28].

According to previous studies, neither NaPi-I nor NaPi-IIa are expressed in lung [4, 13], whereas NaPi-IIb and NaPi-III (particularly Pit-1) are expressed in lung [8, 21, 23]. In lung, NaPi-IIb was reported to be expressed only in rat AT II cells in situ [14], and it was considered to be another specific marker that could be used for studying the spatial and temporal differentiation of AT II cells. Unlike its regulation in the intestine, NaPi-IIb regulation in AT II cells has not been well studied. Glucocorticoids (methylprednisolone) decrease intestinal NaPi-IIb expression [10]. Based on the observation that dex and cAMP treatment help maintain surfactant production in isolated AT II cells, we hypothesized that these agents would influence the expression of other genes such as NaPi-IIb and Pit-1 in an AT II cell culture. From a physiological point of view, the presence of cells upregulating the expression of genes involved in surfactant production is of particular relevance in the perinatal period, and expression of these genes may decrease thereafter [14]. Our data show that NaPi-IIb mRNA is expressed in cultured AT II cells obtained from adult rats, and that dex treatment decreases while cAMP administration stimulates NaPi-IIb mRNA expression in cultured AT II cells. The loss of NaPi-IIb mRNA over time in culture was less pronounced than the loss of SP-C mRNA, but the size of the NaPi-IIb transcripts is noteworthy. Previous studies showed a single band of about 4.0, 4.2, 4.4, or 5.0 kb in intestine or lung from different

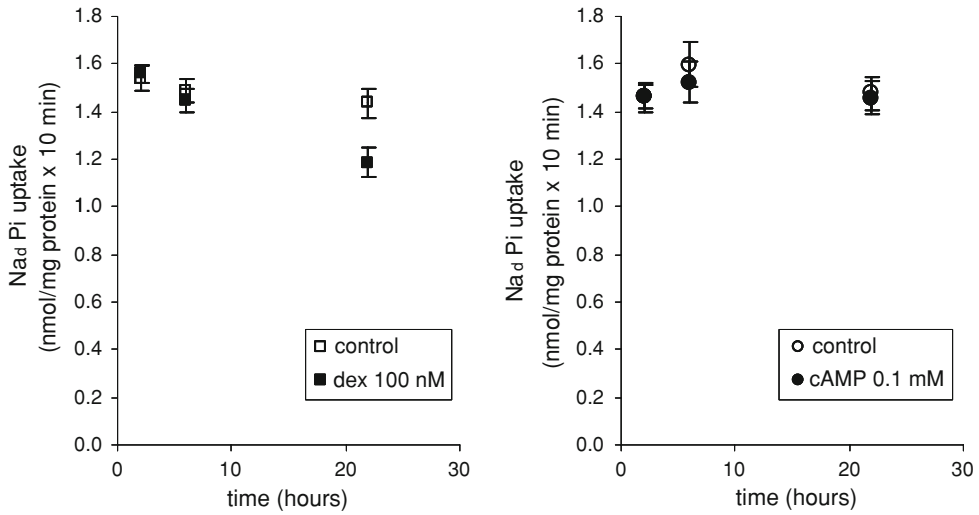

Fig. 6 Na_dPi uptake by AT II cells treated with/without dex or cAMP for 2, 6, and 22 h. Rat AT II cells were cultured in 10% FCS DMEM for 22 h, then exposed to 1 g/l BSA F12 serum-free medium to which dex (to 100 nmol/L final concentration) (*left*, from three separate experiments performed in triplicate) or cAMP (to 0.1 mmol/l final concentration) (*right*, from four separate experiments performed in triplicate) was added for the last 22, 6, or 2 h of culture. Na_dPi uptake was calculated as described in legend to Fig. 5 and "Materials and Methods" section

species [8, 9, 13, 14, 23, 24, 49, 50]. In our present study we observed an approximately 5.0-kb NaPi-IIb mRNA band in rat lung tissue (and also in freshly isolated AT II cells), a band size similar to that of the human lung [24], but two bands of about 4.8 and 4.0 kb in cultured AT II cells (Figs. 2, 3). This suggests distinct NaPi-IIb mRNA transcription or processing in in vivo (in rat lung) and in vitro (in cultured alveolar cells) conditions. Since NaPi-IIb mRNA expression has not been reported in cultured AT II cells before, further study is necessary to confirm this observation.

Pit-1 was originally identified as a retroviral receptor for gibbon ape leukemia virus Glvr-1 [51] and was subsequently found to mediate Pi transport in a Na_d manner [17, 18, 52]. Pit-1 is widely expressed in mammalian tissues [53], including lung. We found much higher Pit-1 mRNA levels in cultured AT II cells and somewhat higher levels in L2 cells than in lung tissue. Higher Pit-1 mRNA levels in AT II than in L2 cells is all the more remarkable since Pit-1 mRNA expression may be related to housekeeping and growth, and L2 cells grow rapidly in contrast to essentially nonreplicating AT II cells. cAMP had no significant effect on Pit 1 mRNA levels in AT II cells, but dex significantly decreased Pit-1 mRNA expression in cultured AT II cells and in L2 cells.

Moreover, we studied Pi uptake and its regulation by dex and cAMP in AT II cell cultures. Most experiments were carried out at a Pi concentration of 0.1 mmol/l, i.e., at a concentration closer to the K_M of the Pi transport system than to plasma Pi concentrations. It is likely that NaPis in the plasma membrane (rather than extracellular Pi) are limiting for Pi uptake by the cells under physiological conditions. Pi uptake in this cell type is Na_d as previously reported by Clerici et al. [30]. These authors found that Pi uptake decreased over time when rat AT II cells were kept for several days in primary culture. According to our findings, Pi uptake in rat AT II cells was slightly decreased by dex, while cAMP treatment had no effect on Pi uptake. It seems that glucocorticoids may induce not only differentiation of AT II cells and surfactant production, but also a progressive increase in caveolin-1 expression and differentiation to AT I cells [54]; the latter may no longer express NaPi-IIb. Decreased Pit-1 mRNA in dex-treated cells is paralleled by corresponding changes in Na_dPi transport. In contrast, cAMP stimulated NaPi-IIb but not Pit-1 mRNA expression and did not significantly alter Pi uptake in AT II cells. Increased Na_dPi transport and Pit-1 expression in response to IGF-I treatment has been found not only in lung but also in bone and muscle cells [21, 36, 55, 56].

The L2 cell line, derived from rat lung, replicates well in culture; however, it has apparently lost expression of most AT II cell-specific genes such as SP-B, SP-C, and NaPi-IIb. Expression of these mRNAs could not be increased to a level detectable by Northern analysis, neither by dex and cAMP nor by growth factors (KGF, VEGF, IGF, TGF-β) (not shown). Na_dPi transport was regulated by dex and IGF-I in a manner comparable to the primary AT II cells; Pit-1 may be responsible for Pi uptake in this cell type.

Transcripts encoding NaPi-IIb and Pit-1 could be detected in the cultured cells as previously in rat lung and intestine and in brain, heart, bone, lung, intestine, and osteoblastic cells, respectively [21]. Unfortunately, however, our attempts to identify NaPi-IIb and Pit-1 in plasma membranes of cultured AT II cells by western analysis using available antibodies were unsuccessful, so far. Although we have been able to detect bands at the corresponding expected sizes in plasma membranes prepared from rat gut and lung (but not from brain, heart, and kidney) (108 kDa) and in plasma membranes from osteoblastic cells (85 kDa) [21], respectively, we failed to identify corresponding bands in the cultured AT II cells. It appears that these cells no longer expressed the protein at a level sufficient for detection or that the sensitivity of our analysis was too low. The latter may well be the case for Pit-1, where it has been notoriously difficult to identify the protein by antibodies, apart from a few exceptions [20, 53, 56].

Because AT II cells need sufficient Pi for surfactant synthesis, an apical membrane-distributed NaPi-IIb may be considered to play an important role in Pi uptake and reutilization of Pi in AT II cells [13]. Indeed, impaired NaPi-IIb function appears to play a role in the pathogenesis of PAM; in this disorder, recycling of Pi accumulating from degraded surfactant phospholipids, i.e., its clearance from the alveolar space, appears to be disturbed [16]. Some NaPi-IIb mRNA expression is maintained in our rat cell culture model that allowed us, for the first time, to demonstrate upregulation of NaPi-IIb mRNA by cAMP. However, this was not associated with an increase in Pi uptake in the cultured cells. Dex decreased NaPi-IIb and Pit-1 mRNA expression as well as Pi uptake concomitantly. Thus, dex has opposite effects on PS synthesis and Pi uptake, whereas IGF-I stimulates Pit-1 mRNA and Pi uptake in AT II cells. Given that we could not detect NaPi-IIb and Pit-1 proteins and link them functionally to Pi uptake, it remains unclear whether and to what extent they can account for Pi transport in AT II cells.

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