Melatonin Modulates Acid/Base Transport in Human Pancreatic Carcinoma Cells

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Key Words

Melatonin • Pancreas • Intracellular pH • Solute carrier • Bicarbonate secretion • Melatonin receptor

Abstract

Melatonin was found to improve pancreatic organ function in diseased animals. To study whether pancreatic bicarbonate secretion is stimulated by melatonin, investigations were done in two human ductal pancreatic adenocarcinoma cell lines MIA PaCa-2 (MIA) and PANC-1 (PANC). Using the fluorescence pH-sensor BCECF-AM, we monitored melatonin effects on basal intracellular pH (pH_i), and on pH_i recovery after intracellular alkalinization produced by the removal of extracellular HCO_3^-/CO_2 . Exposure to 1 μM melatonin for 24 hrs and presence of the indoleamine during the experiment increases the basal pH_i . Moreover, pH_i recovery and HCO_3^- secretion are facilitated after the alkaline load. These findings are in line with the observed increase in mRNA expression of the Na^+/HCO_3^- -cotransporter SLC4A4b for the uptake and the Cl^-/HCO_3^- -exchanger SLC26A6

for the secretion of HCO_3^- . The reduction in Na^+/H^+ -exchanger SLC9A1 mRNA would favor pH_i recovery after alkalinization, but it does not explain the initial increase in pH_i . This controversial effect and the requirement for continuous presence of melatonin throughout the experiment suggest that non-transcriptional signalling may contribute to the effects of melatonin on acid/base movements. In summary, we show a stimulatory effect of melatonin on bicarbonate secretion in the pancreatic cancer cell lines which may help to prevent duodenal damage.

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Introduction

Melatonin, originally thought to be produced and secreted exclusively by the pineal gland, is present in the gastrointestinal tract at high concentrations, which, during daytime, exceed serum levels by 10 to 100-fold [1, 2]. Gastrointestinal melatonin is partly taken up from the circulation, but a large amount of melatonin itself or its

precursors serotonin and tryptophan are derived from dietary sources [3, 4]. Expression of the two melatonin synthesizing enzymes, arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) was detected in various gastrointestinal organs including pancreas, although pancreatic melatonin synthesis has not been proven so far [5].

Melatonin was suggested to have beneficial effects in various gastrointestinal organs. In the stomach, melatonin was found to inhibit acid secretion [6, 7], and it induces duodenal HCO_3^- secretion in response to gastric acid [8]. Studies in animals showed that melatonin stimulates pancreatic enzyme secretion and improves the outcome of experimental pancreatitis [9-11]. Favorable effects of melatonin on pancreatic function have been related to its antioxidative effects [12-14] and, in line with findings in other gastrointestinal organs, its ability to regulate ion transport and to stimulate epithelial regeneration might contribute [15].

Quantitatively, the pancreatic duct epithelium has the highest HCO_3^- secretion capacity with concentrations reaching up to 150 mM HCO_3^- in the pancreatic fluid. This efficient secretion of HCO_3^- is essential to neutralize the acidic chyme and dissolve secreted enzymes thereby preventing duodenal ulcers [16].

Transcellular transport of membrane impermeable HCO_3^- is facilitated by integral membrane transport proteins, such as members of the SLC family. In the pancreatic ductal epithelial cells, HCO_3^- secretion is dependent on the basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter (SLC4A4b) [17], through which HCO_3^- enters the cells from the blood, and on the uptake of CO_2 , which directly permeates into the cell interior, where it is rapidly converted into HCO_3^- and H^+ by the carbonic anhydrase. While H^+ are removed by the basolateral Na^+/H^+ exchanger (SLC9A1), HCO_3^- extrusion across the apical membrane is mediated by two $\text{Cl}^-/\text{HCO}_3^-$ exchangers from the SLC26A family, namely the SLC26A6 and SLC26A3 [19]. SLC26A6 has a major role in luminal HCO_3^- secretion and it is proposed that this exchanger is stimulated by the activity of the ATP-binding cassette transporter ABCC7, the cystic fibrosis transmembrane conductance regulator CFTR [20].

Analogous to its stimulatory effect on bicarbonate secretion in the duodenum [21], we are interested whether melatonin enhances HCO_3^- secretion in pancreatic ducts as well. It is also remarkable to note that melatonin was found to alter membrane transport properties in other cells as well. For example, in kidney cells, melatonin modulates water movements by stimulation of Na^+/K^+ ATPase

activity via a mechanism involving actin binding [22, 23].

To stimulate pancreatic bicarbonate secretion, melatonin could modulate solute transporter activity and/or alter their expression. For this purpose, the indoleamine might act through binding and activation of its G-protein coupled melatonin receptors MT1 and MT2 or in a receptor independent way [24-27]. At the molecular level, activation of MT1 and MT2 inhibits forskolin-stimulated cAMP formation via a pertussis toxin sensitive G_i protein in various cells and tissues. On the other hand, stimulation of cAMP synthesis via a Ca^{++} -calmodulin dependent pathway requiring MT1 activation [28] or being independent on the G-protein coupled receptors [29] had been found in different cell lines under certain conditions. Additionally, melatonin was found to modulate gene transcription via nuclear receptors, e.g. through retinoid orphan receptor (ROR) α activation [30]. While receptor activation requires nanomolar concentrations of the indoleamine, at micromolar concentrations, melatonin is a powerful antioxidant [31]. Such high concentrations were found to be reached, e.g. in lymphocytes [32]. It acts as a direct scavenger of free radicals and it also acts in a non-scavenging way. For example, it activates and induces the expression of antioxidative enzymes and inhibits pro-oxidant enzymes, e.g. nitric oxide synthase [31, 33].

Through these different mechanisms, melatonin could improve the function of the exocrine pancreas. In any case, by influencing ion homeostasis and inducing pancreatic bicarbonate secretion, together, with its direct stimulating effect on duodenal alkaline secretion [8], melatonin could offer a powerful protection against acid-induced ulcers in the duodenum.

These considerations prompted us to study the influence of melatonin on basal intracellular pH (pH_i), and on the rate of HCO_3^- secretion in two pancreatic carcinoma cell lines, namely MIA PaCa-2 (MIA) and PANC-1 (PANC) cells, both, derived from human ductal pancreatic adenocarcinomas. These cell lines offer a good model for the investigation of melatonin effects on acid/base movements, as they express different amounts of solute transporters SLC4A4b and SLC26A6 for bicarbonate uptake and secretion, respectively, as well as the Na^+/H^+ exchanger SLC9A1.

Materials and Methods

Materials

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), nigericin, choline chloride, forskolin, tetramethylammonium hydroxide (TMA-OH),

melatonin, gluconic acid, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM), bisbenzimidazole, saponin, Tris(hydroxymethyl)aminomethane (TRIS), ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), Nonidet NP-40, and a combined phosphatase/protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). All other chemicals were from Merck (Darmstadt, Germany). RPMI 1640 cell culture medium without phenol red, penicillin/streptomycin and charcoal-treated fetal bovine serum (FBS) were from PAN Biotech (Aldenbach, Germany). Fetal bovine serum (FBS) and trypsin/EDTA were from GIBCO, Invitrogen Corp. (Grand Island, NY).

Cell culture

MIA PaCa-2 (ATCC CRL 1420) and PANC-1 (ATCC CRL 1469) cells were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). Both cell lines were maintained in RPMI 1640 tissue culture medium without phenol red supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Cells were cultivated under standard conditions at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Media were changed every second day and cells were passaged by trypsinization twice a week. For isolation of total RNA, cells were treated with 1 µM melatonin (1 mM stock in DMSO) for 24 h in RPMI 1640 medium supplemented with 2.5% charcoal treated FBS and antibiotics. For intracellular pH measurements, two days before the experiment, cells were transferred to 6-well plates and grown on glass coverslips. 24 h before the measurements, cells were treated with 1, 0.5 and 0.1 µM melatonin, respectively. Control experiments were performed with the vehicle only (0.1% DMSO).

Buffers for pH experiments

Bicarbonate-free bath solution contained (mM): NaCl 135, KCl 4.7, MgSO₄ 1, KH₂PO₄ 1.2, CaCl₂ 1.5, HEPES 10, glucose 5, titrated to pH 7.4 at 37°C with NaOH. In the bicarbonate buffered solution, HEPES was replaced by 25 mM NaHCO₃ and equilibrated with 5% CO₂ at 37°C.

Intracellular pH (pH_i) measurements

Intracellular pH was measured using the fluorescent intracellular sensor BCECF [34]. In brief, BCECF was loaded into cells in the form of its tetraacetoxymethylester derivative (BCECF-AM) (12 µM) by incubation for 10-20 min at 37°C. After washing for 10 min at 37°C in the BCECF-free bicarbonate solution, the cell containing glass coverslips were transferred into a thermostated (37°C) perfusion chamber placed on the stage of an Axiovert (Zeiss, Jena, Germany) inverted microscope. The microscope was equipped with a microfluorometer (Photon Technology Instruments, Monmouth Junction, NJ) for continuous dual wavelength excitation photometry.

Intracellular pH was measured in single cells as the ratio of emission intensities at 530 nm after excitation at 495 nm (pH_i and concentration sensitive) and 440 nm (only concentration

sensitive), respectively. Data were collected at 50 Hz chopping frequency and averaged every 2 sec. After each experiment, internal dye calibration was performed by superfusing the cells with nigericin (12 µM) at pH 6.8 and 7.6, respectively.

Measurements of pH recovery rate after bicarbonate load

Cells were first superfused for 60 sec with a 25mM HCO₃⁻, 5% CO₂ containing solution, which results in intracellular equilibration of HCO₃⁻ according to pH_i. Acute removal of CO₂/HCO₃⁻ from the superfusion medium by switching to the HEPES-buffered bicarbonate-free solution, results in a sudden depletion of intracellular CO₂ and intracellular alkalization. Thereafter, intracellular pH recovers from this alkaline load towards the initial value by HCO₃⁻ extrusion [35].

Data analysis and statistics

To avoid systematic errors in comparing one experimental condition with control, we performed the respective experiments on the same day, from one set of cell cultures. Data were acquired from single cells. They are given as mean values ± SD (n=number of experiments). $\delta\text{pH}/\delta t = \text{U/min (sec 100 - sec 160)}$. Differences between grouped experiments are evaluated by the one-tailed unpaired Students t-test.

Conventional and quantitative real-time RT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Paisley, Scotland). cDNAs were prepared according to standard methods. Intron-spanning primers were used for conventional RT-PCR using human gallbladder and retina as positive controls for MT1 and MT2, respectively. MT1 (286 bp): forward: 5'-TCC TGG TCA TCC TGT CGG TGT ATC-3', reverse: 5'-CTG CTG TAC AGT TTG TCG TAC TTG-3' [36]; MT2 (322 bp): forward: 5'-TCC TGG TGA TCC TCT CCG TGC TCA-3', reverse: 5'-AGC CAG ATG AGG CAG ATG TGC AGA-3' [24]. Each cycle of PCR consisted of 30 sec at 94°C, 1 min at 65°C and 1 min at 72°C. The primer sequences for the PCR of the transporters are as follows: SLC26A6 (406 bp): forward: 5'-CAG CTG TGC AGG TCT TCG TCT C-3', reverse: 5'-GCAAC CCA ACC ACA GCG ATG-3'; SLC4A4b (400 bp): forward: 5'-CCC TGA CTG AAG TTC CTG CTG TGC C-3', reverse: 5'-AGT CCA CCA CAG AAC CGT CCA G-3'; SLC9A1 (249 bp): forward: 5'-CCA GCT CAT TGC CTT CTA CC-3', reverse: 5'-TGT GTC TGT TGT AGG ACC GC-3' [37]; SLC26A3 (406 bp): forward: 5'-TCC AGT GCC CAT TCC AAT CG-3', reverse: 5'-CAC GAT GAT GGC ACC AAT AAG C-3'. PCR conditions: SLC26A6: 40/35 cycles of 94°C 15 sec, 60°C 30 sec, 72°C 1 min. SLC4A4b: 40 cycles of 94°C 15 sec, 64°C 30 sec, 72°C 1 min. SLC9A1: 35 cycles of 94°C 30 sec, 58°C 30 sec, 72°C 1 min. SLC26A3: 40 cycles of 94°C 30 sec, 59°C 1 min, 72°C 1 min.

β-actin served as an endogenous control. For negative controls, the PCR reaction was performed without a template or with RNA prior to reverse transcription. Amplified cDNA was visualized by ethidium bromide staining on 2% agarose gels on a Herolab gelscanner (Wiesbach, Germany).

For relative quantitation of MT1 and MT2 mRNA, respectively, gene-specific oligonucleotide primer pairs and oligonucleotide probes, respectively, labeled with 6-

carboxyfluorescein (FAM) and the quencher 6-carboxytetramethylrhodamine (TAMRA), were designed using the Primer Express Software (National Bioscience, Plymouth, MN). The following primers and probes were applied: MT1 forward: 5'-CGG TGT ATC GGA ACA AGA AGC T-3', MT1 reverse: 5'-AGG TCT GCC ACC GCT AAG C-3', MT1 probe: 6-FAM-TCA CCA CAA AGA TGT TTC CTG CGT TCC T-TAMRA; MT2 forward: 5'-TGC TCA GGA ACC GCA AGC-3', MT2 reverse: 5'-AGG TCA GCC AAT GCC AGA CT-3', MT2 probe: 6-FAM-ACC AAG AAC AAA TTA CCT GCG TTC CGG-TAMRA. For relative quantitation of pancreatic solute transporters, TaqMan® Gene Expression Assays (Applied Biosystems, AB, Foster City, CA) were applied: SLC26A6: Hs00370470_m1, SLC4A4: Hs00186798_m1, SLC9A1: Hs00384604_m1. All mRNA levels were normalized to ribosomal 18S expression. The PCR reactions were performed on the ABI PRISM 7700 Sequence Detector (AB). Respective mRNA levels were calculated using the relative standard curve method (AB, User Bulletin 2).

Treatments with either melatonin or the solvent (0.1% DMSO) were performed for each cell line and RNAs were isolated and reverse transcribed. Subsequent PCRs were performed in triplicates. For quantitative PCR, four plates, in which samples were done at least in triplicates, were independently analyzed for each target. Differences were evaluated by an unpaired Student's t-test. Differences are considered significant if $p < 0.05$.

Measurement of total cellular cAMP

96-well plates containing 2×10^4 cells/well were first pretreated for 24 hrs with 2.5% charcoal stripped FBS in RPMI 1640 medium, before 1 μ M melatonin was added for 5 min and 24 hrs, respectively. The subsequent cell lysis and the enzyme immunoassay (EIA) were performed using the cAMP Biotrak EIA System (Amersham Biosciences UK Ltd, Buckinghamshire, UK). In brief, following the addition of the lysis reagent, a microscopic evaluation of cell lysis was done with Trypan blue. Thereafter, 100 μ l aliquots were transferred to the antibody coated 96-well plate for the assay. Preparation of working standards and the assay procedure were carried out according to the manufacturers' protocol and the optical density was determined at 450 nm within 30 min. Experiments were done in triplicates.

Results

Effects of melatonin on pH_i in MIA PaCa-2 (MIA) and PANC-1 (PANC) cells

For the evaluation of melatonin effects on pH_i , MIA and PANC cells were treated with 1 μ M of the hormone under different experimental conditions (3 groups). In the MEL group, cells were exposed to melatonin during the pH measurement. Cells from the inc group were pretreated with melatonin for 24 hrs, but the hormone

was absent during measurements. In the MEL/inc group, cells were both, preincubated and exposed to melatonin throughout the experiment.

As demonstrated in Fig. 1 (A-D), in absence of melatonin (control), the basal pH_i in MIA was 7.16 ± 0.03 and in PANC cells 7.1 ± 0.06 . To study the effect of melatonin on bicarbonate secretion, NaHCO_3 was replaced by HEPES in the external buffer and CO_2 was omitted. This caused a rapid increase in the pH_i by 0.24 units (to 7.40 ± 0.04) in MIA and by 0.39 units (to 7.49 ± 0.07) in PANC cells. During the following stimulation of bicarbonate extrusion, the recovery rate of pH_i in MIA was slower ($\delta pH/\delta t = -0.064 \pm 0.012$ /min) than in PANC cells ($\delta pH/\delta t = -0.09 \pm 0.04$ /min).

As shown in Fig. 1, A-D, melatonin shifted basal pH_i towards a more alkaline value. This change in basal pH_i was significant, if MIA and PANC cells were both, pretreated for 24 hrs with the hormone and exposed to 1 μ M melatonin during measurements (MEL/inc). The increase of pH_i was more pronounced in MIA cells, in which pH_i rose to 7.34 ± 0.07 ($p < 0.01$ vs. control), while in PANC cells, pH_i rose to 7.21 ± 0.07 ($p < 0.05$ vs. control).

In MEL/inc treated cells, the rate of recovery of pH_i after an alkaline load was increased. In MIA cells, the rate was strongly accelerated from $\delta pH/\delta t -0.064 \pm 0.012$ to $\delta pH/\delta t -0.09 \pm 0.02$ ($p < 0.01$), while in PANC cells, the increase in the rate was not significant ($\delta pH/\delta t -0.09 \pm 0.04$ to $\delta pH/\delta t -0.11 \pm 0.04$, n.s. (Fig. 1,C,D).

Remarkably, under both, MEL and inc conditions, the basal pH_i and the recovery rate after alkaline load were not significantly altered in MIA (Fig. 1A) and in PANC cells (Fig. 1B), respectively.

The alkalinization of basal pH_i in MEL/inc treated cells could be either due to bicarbonate accumulation through increased uptake of bicarbonate via the sodium-bicarbonate cotransporter SLC4A4b or due to increased extrusion of H^+ via the Na^+/H^+ exchanger SLC9A1. Additionally, inhibition of luminal bicarbonate extrusion via the $\text{Cl}^-/\text{HCO}_3^-$ exchanger SLC26A6 could contribute to the increase in pH_i .

To gain insight into the contribution of individual transporters, ion substitution studies were done, in which in the extracellular medium, i) Na^+ was replaced by choline and ii) Cl^- by gluconate (Fig. 1 E,F).

i) In the absence of extracellular Na^+ , the basal pH was lower as compared to MEL/inc data in presence of Na^+ (7.08 ± 0.03 vs. 7.34 ± 0.07 ; $p < 0.05$ in MIA and 7.01 ± 0.01 vs. 7.21 ± 0.07 ; $p < 0.05$ in PANC). It was comparable to the more acidic pH_i values in untreated

Fig. 2. Effect of melatonin on solute carrier expression. Left panels: Relative quantitation (arbitrary units) of SLC26A6, SLC4A4b and SLC9A1 mRNA expression in untreated (control) and 24 hrs melatonin treated (+ MEL) MIA and PANC cells by TaqMan PCR using either control cell line as reference. The following amounts of cDNA for the solute transporters were applied: 0.05 ng, 20 ng and 0.1 ng for SLC26A6, SLC4A4b and SLC9A1, respectively. Respective mRNA levels were calculated using the relative standard curve method (AB, User Bulletin 2). Cells were treated either with melatonin or the solvent (0.1% DMSO) before RNA isolation. Data from individual experiments done at least in triplicates are shown. Differences were evaluated by an unpaired Students t-test. (* $p < 0.05$, ** $p < 0.01$). Right pictures: mRNA expression of the solute carriers SLC26A6 (406 bp), SLC4A4b (400 bp) and SLC9A1 (249 bp) in untreated and 24 hrs melatonin treated MIA + MEL and PANC + MEL cells normalized to beta-actin. In the negative control (- Control), the PCR reaction was performed in absence of a template.

ii) In MEL/inc treated cells in Cl^- -free medium the basal pH_i was 7.33 ± 0.05 in MIA and 7.31 ± 0.04 in PANC cells. These values were not different from values of the MEL/inc treated cells in chloride containing normal medium (Fig. 1C,D), in which the initial pH_i was 7.34 ± 0.07 in MIA MEL/inc and 7.21 ± 0.07 in PANC MEL/inc. The lack of Cl^- dependence makes it unlikely that the melatonin effect on the initial pH_i is caused by reduced activity of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger SLC26A6.

In MEL/inc cells, Na^+ (i) and Cl^- (ii) free conditions also affected the rate of pH_i recovery after an alkaline load:

i) In absence of Na^+ , the recovery of the pH_i to baseline levels was decelerated. A recovery rate of $\delta \text{pH}/\delta t = -0.062 \pm 0.022$ ($p < 0.05$) was observed in MIA and of $\delta \text{pH}/\delta t = -0.05 \pm 0.02$ ($p < 0.01$) in PANC cells, indicating that a Na^+ -dependent mechanism of HCO_3^- extrusion (e.g. Na^+ -dependent HCO_3^- uptake coupled to Cl^- -dependent

Fig. 3. Measurement of cAMP in MIA and PANC cells. The formation of cAMP was determined by EIA in cells treated with 0.1% DMSO, with 1 μ M melatonin, 1 μ M forskolin, and with both, melatonin and forskolin for 5 min (white column) and 24 hrs (black column), respectively. (n=3; p<0.05).

MIA	n	pH _i	pH (60 sec)	δ pH/ δ t (U/min)
Control	7	7.16 $\pm$ 0.03	7.40 $\pm$ 0.04	-0.064 $\pm$ 0.012
MEL/inc [1 μ M]	10	7.34 $\pm$ 0.07**	7.52 $\pm$ 0.09**	-0.09 $\pm$ 0.02**
MEL/inc [0.5 μ M]	10	7.09 $\pm$ 0.06	7.32 $\pm$ 0.08	-0.075 $\pm$ 0.018
MEL/inc [0.1 μ M]	9	7.13 $\pm$ 0.07	7.37 $\pm$ 0.06	-0.079 $\pm$ 0.034

Table 1. Effects of different melatonin doses on basal pH_i and pH_i recovery after alkaline load in MIA cells. For experimental conditions see legend to Figure 1. Melatonin doses of 1, 0.5 and 0.1 μ M were applied for the preincubation and during the experiment, respectively. (Means $\pm$ SD, p vs. control; ** p<0.01)

HCO₃⁻ extrusion) also contributes to the recovery of pH_i after an alkaline load.

ii) Removal of extracellular Cl⁻ prevented the recovery of the pH_i to baseline values in MIA (δ pH/ δ t = -0.037 $\pm$ 0.014, p<0.01) and PANC cells (δ pH/ δ t = -0.03 $\pm$ 0.01, p<0.01), suggesting that Cl⁻-dependent HCO₃⁻ extrusion via SLC26A6 is the major mechanism of HCO₃⁻ secretion in MEL/inc treated cells. As stated below, expression of the other Cl⁻/HCO₃⁻ exchanger SLC26A3 was hardly detectable.

Studies were done with two additional melatonin doses (0.1 and 0.5 μ M) to establish a dose dependency for the effect of MEL/inc treatment on intracellular pH

regulation (Table 1). These studies were carried out in MIA cells, in which significant effects on pH_i regulation were observed for MEL/inc (1 μ M) treatment. Although 0.1 and 0.5 μ M melatonin had a tendency to stimulate pH_i recovery after bicarbonate load, these effects did not reach the level of significance.

Influence of melatonin on the mRNA expression of pancreatic solute transporters SLC26A6, SLC4A4b, SLC9A1

As assessed by conventional PCR, substantial amounts of mRNA of the solute transporters SLC26A6 and SLC9A1 are expressed in MIA and PANC cells,

while SLC4A4b mRNA is near the detection limit in MIA cells (Fig. 2). Quantitative PCR revealed that the mRNA expression levels of SLC26A6 and SLC9A1 are comparable in MIA and in PANC cells, while SLC4A4b mRNA levels are 6-fold higher in PANC cells ($p < 0.05$) (Fig. 2).

Treatment with 1 μM melatonin for 24 hrs (MEL) increased mRNA expression of the HCO_3^- transporters SLC26A6 and SLC4A4b in MIA cells. However, it reduced the Na^+/H^+ exchanger SLC9A1 mRNA. Quantitative PCR revealed a 2.2 ($p < 0.01$) and a 2.3-fold ($p < 0.05$) increase of SLC26A6 and SLC4A4b mRNA, respectively in MIA cells. In contrast, a 40% reduction of SLC9A1 mRNA was observed in both cell lines ($p < 0.05$).

Both, conventional and quantitative PCR revealed that the second $\text{Cl}^-/\text{HCO}_3^-$ transporter, namely SLC26A3, which is expressed in the apical membrane of normal pancreatic ductal epithelial cells [38] is near the detection limit in the two cell lines. Additionally, the splice variant SLC4A4a, also present in normal pancreatic tissue [17], is not detectable in these cells (data not shown). Therefore, no further studies on these transporters were done.

Melatonin inhibits forskolin induced cAMP formation in MIA and PANC cell lines

As previous studies showed that ion movements in pancreatic duct cells are coupled to cAMP/PKA activation, we investigated whether melatonin might alter cAMP levels in the pancreatic carcinoma cell lines (Fig. 3). We applied 1 μM melatonin, as this dose was effective to alter solute movements in these cell lines. As assessed by an enzyme immunoassay (EIA), basal cAMP levels were 2-fold higher in PANC than in MIA cells and were not affected by short (5 min) and long (24 hrs) term treatment with melatonin. However, melatonin reduced forskolin-induced formation of cAMP, an effect which was previously described to be mediated via activation of G-protein coupled melatonin receptors MT1/MT2 [25]. After 24 hrs of treatment with 1 μM forskolin, cAMP was increased by 3.7-fold in MIA and 2.6-fold in PANC cells. Incubation with 1 μM melatonin for 24 hrs significantly reduced the forskolin-stimulated increase in intracellular cAMP in both cell lines (by 50% and 30 % in MIA and PANC cells, respectively, $p < 0.05$).

Further RT-PCR studies showed that both cell lines express MT1, but not MT2 mRNA (Fig. 4A) and protein (Fig. 4B). Higher MT1 mRNA levels were observed in less sensitive PANC cells (11.9 ± 2.34 -fold higher in PANC

Fig. 4. Expression of melatonin receptors MT1 and MT2 in pancreatic carcinoma cells. (A) mRNA expression of G-protein coupled melatonin receptors MT1 and MT2 in untreated (MIA and PANC) and 24 hrs melatonin treated (MIA/PANC + MEL) pancreatic carcinoma cell lines. The amplicon sizes were 286 bp for MT1 and 322 bp for MT2. cDNA from gallbladder and retina served as positive controls (+ Control) for MT1 and MT2, respectively. (B) Western blot analysis of MT1 and MT2 protein expression in untreated MIA and PANC cells, and 24 hrs melatonin treated cells (MIA/PANC + MEL). The MT1 antibody recognized a band at approximately 40 kDa.

than in MIA cells) and melatonin treatment (1 μM for 24 hrs) caused a 30-40% down-regulation of MT1 mRNA expression.

Discussion

Our data show that 1 μM melatonin stimulates HCO_3^- secretion in MIA and PANC pancreatic carcinoma cell lines. Stimulation of HCO_3^- secretion might contribute to the beneficial effects of the indoleamine in experimental pancreatic disease reported from animal studies [5].

Fig. 5. Effects of melatonin on the expression of transporters for HCO_3^- secretion in a pancreatic cancer cell. HCO_3^- secretion in a pancreatic ductal cancer cell is mediated by members of the SLC family. HCO_3^- enters the cells via the $\text{Na}^+/\text{HCO}_3^-$ cotransporter SLC4A4b and it is also derived from CO_2 , which directly permeates into the cell interior, where it is rapidly converted to HCO_3^- and H^+ by the carbonic anhydrase. While H^+ are removed by the Na^+/H^+ exchanger SLC9A1, HCO_3^- extrusion occurs via the $\text{Cl}^-/\text{HCO}_3^-$ exchanger SLC26A6. MEL increases ($\uparrow$) mRNA expression of SLC4A4b for HCO_3^- uptake and SLC26A6 for HCO_3^- secretion, while mRNA expression of the Na^+/H^+ exchanger SLC9A1 mRNA is reduced ($\downarrow$). The second $\text{Cl}^-/\text{HCO}_3^-$ exchanger SLC26A3 is not detectable in the pancreatic cell lines MIA and PANC (dotted line). The ATP-binding cassette transporter ABCC7, the cystic fibrosis transmembrane conductance regulator CFTR, recycles Cl^- [17].

In humans, melatonin is frequently applied as an “over the counter drug” against disorders like jet lag, insomnia, seasonal depression [39] and it is used in alternative cancer therapy [40]. For the latter, doses as high as 80 mg are given and serum melatonin concentrations up to 10.000-fold higher than the usual night time peak concentration of 0.26 nM were reported [41]. In that case, serum concentrations of approx. 1 μM , which correspond to the melatonin dose effective in the two pancreatic cancer cell lines, could be expected.

Using the pH_i sensor BCECF-AM, we demonstrate that, particularly in MIA cells, melatonin increases basal pH_i and, after an alkaline load, it stimulates base extrusion, thereby accelerating the recovery of pH_i to initial values in melatonin pretreated cells in the presence of the indoleamine. Under these conditions, an increase in mRNA expression of SLC4A4b for HCO_3^- uptake and SLC26A6 for HCO_3^- secretion is observed, while mRNA expression of the Na^+/H^+ exchanger SLC9A1 mRNA is reduced (see Fig. 5). Contribution of SLC26A3 on HCO_3^-

secretion can be excluded as its RNA was not detectable in both cell lines.

The increase in basal pH_i by melatonin is Na^+ -dependent and goes along with an enhanced mRNA expression of the $\text{Na}^+/\text{HCO}_3^-$ cotransporter SLC4A4b, whereas stimulated HCO_3^- extrusion strongly depends on extracellular Cl^- . Therefore, the increased mRNA expression of the $\text{Na}^+/\text{HCO}_3^-$ cotransporter SLC4A4b which is paralleled by an increased expression of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger SLC26A6 mRNA can at least partly account for enhanced HCO_3^- movements observed in this study.

Moreover, the correlation appears to be quantitative in that the twofold higher induction of SLC4A4b and SLC26A6 mRNA in MIA as compared to PANC cells is linked to the more pronounced effects of the hormone on basal pH_i and on HCO_3^- secretion in the former cell line. Not entirely concordant with these observations, we found that expression of the Na^+/H^+ exchanger SLC9A1 mRNA is reduced by melatonin, which, at the functional level,

would be expected to acidify initial basal pH_i . If melatonin affects acid/base movements only through altered transporter expression, an initial intracellular acidification caused by decreased expression of SLC9A1 would be expected. Otherwise, enhanced bicarbonate influx via the increased SLC4A4b should counteract this acidification. However, this does not seem to be sufficient, as the observed rise in initial pH_i requires the presence of melatonin in addition to melatonin pretreatment. This suggests that melatonin has additional non-genomic effects on acid/base transport as well.

On the other hand, reduced expression of SLC9A1 mRNA would facilitate pH_i recovery to initial values after alkaline load produced by removal of extracellular $\text{HCO}_3^-/\text{CO}_2$. Therefore, melatonin seems to have different effects on SLC9A1. In fact, recent data from studies in mouse pancreatic acinar cells revealed that SLC9A1 activity is coupled to the function of the intracellular carbonic anhydrase [42], which vice versa, depends on the concentration of $\text{HCO}_3^-/\text{CO}_2$ in the extracellular medium [43]. Through altered activity of this enzyme, divergent effects of melatonin on SLC9A1 activity in the absence and presence of $\text{HCO}_3^-/\text{CO}_2$ might be explained.

Furthermore, the $\text{Cl}^-/\text{HCO}_3^-$ exchanger is activated by an increase in intracellular Ca^{++} which induces Cl^- influx via apical membrane Cl^- channels. This leads to an increased HCO_3^- secretion and enhances pH_i recovery after alkaline load [35]. This is concordant with our data, which show that the recovery is sensitive to the removal of Cl^- .

In line with these findings, alterations in intracellular Ca^{++} by melatonin were reported to increase HCO_3^- secretion in isolated duodenal enterocytes. In these cells, melatonin induced the release of Ca^{++} from intracellular stores and stimulated Ca^{++} influx [44].

Although this was attributed to MT2 activation in enterocytes, we can exclude involvement of MT2 on the observed acid/base movements in both pancreatic cancer cell lines, as MT1 was the only MT isoform detectable in MIA and PANC cells. However, the following observations suggest that MT1 is unlikely to modulate acid/base transport in these cell lines: i) The effective dose of 1 μM to modulate ion movements in the pancreatic

cancer cell lines is clearly above the K_d of 30-160 pM for ^{125}I -iodomelatonin and 1 nM melatonin for MT1 activation [45]. ii) MT1 expression levels are lower in MIA than in PANC cells, while melatonin effects on ion transport are more pronounced in MIA cells. iii) Treatment with 1 μM melatonin for 24 hrs down-regulates MT1 expression, which would diminish the effectiveness of MT1 mediated signaling. This is in accordance with data from MT1 overexpressing CHO cells, in which longer exposure to the hormone not only caused a down-regulation of the receptor but also decreased the affinity for the hormone [27]. iv) In both cell lines, we found that melatonin inhibits forskolin-induced cAMP formation. Since it has previously been shown that in PANC cells, the forskolin-induced cAMP formation stimulates HCO_3^- extrusion [35], inhibition of cAMP formation, as observed in this study, should rather inhibit HCO_3^- secretion. Because the opposite is true in our studies, we thus conclude that cAMP signalling is not a means of altering ion transport activities in the pancreatic cancer cell lines.

Taken together, our data on the melatonin-induced stimulation of bicarbonate secretion in the two pancreatic cancer cell lines provide the first evidence for a transcriptional effect of melatonin on ion transport proteins although non-genomic effects also contribute. Increased bicarbonate secretion in the pancreatic ducts may greatly add to the beneficial effects of melatonin observed in the gastrointestinal tract.

Abbreviations

PANC (PANC-1); MIA (MIA PaCa-2); pH_i (intracellular pH); MT1 (melatonin receptor 1), MT2 (melatonin receptor 2).

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