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Renal Proximal Tubule Na,K-ATPase is Controlled by CREB Regulated Transcriptional CoActivators as well as Salt Inducible Kinase 1

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Abstract

Sodium reabsorption by the kidney is regulated by locally produced natriuretic and anti-natriuretic factors, including dopamine and norepinephrine, respectively. Previous studies indicated that signaling events initiated by these natriuretic and anti-natriuretic factors achieve their effects by altering the phosphorylation of Na,K-ATPase in the renal proximal tubule, and that Protein Kinase A (PKA) and Calcium mediated signaling pathways are involved. The same signaling pathways also control the transcription of the Na,K-ATPase β subunit gene *atp1b1* in renal proximal tubule cells. In this report, evidence is presented that 1) both the recently discovered cAMP-Regulated Transcriptional Coactivators (CRTCs), and Salt Inducible Kinase 1 (SIK1) contribute to the transcriptional regulation of *atp1b1* in renal proximal tubule (RPT) cells, and 2) that renal effectors including norepinephrine, dopamine, prostaglandins and sodium play a role. Exogenously expressed CRTCs stimulate *atp1b1* transcription. Evidence for a role of endogenous CRTCs includes the loss of transcriptional regulation of *atp1b1* by a dominant negative CRTC, as well as by a CREB mutant, with an altered CRTC binding site. In a number of experimental systems, SIK phosphorylates CRTCs, which are then sequestered in the cytoplasm, preventing their nuclear effects. Consistent with such a role of SIK in primary RPT cells, *atp1b1* transcription increased in the presence of a dominant negative SIK1, and in addition, regulation by dopamine, norepinephrine and monensin was disrupted by a dominant negative SIK1. These latter observations can be explained, if SIK1 is phosphorylated and inactivated in the presence of these renal effectors. Our results support the hypothesis that Na,K-ATPase in the renal proximal tubule is regulated at the transcriptional level via SIK1 and CRTCs by renal effectors, in addition to the previously reported control of the phosphorylation of Na,K-ATPase.

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Keywords

renal proximal tubule; dopamine; norepinephrine; prostaglandins; Na, K-ATPase; transcription; CRTC; SIK1

1. Introduction

The kidney is a major regulator of blood pressure, and must respond rapidly to changes in Na^+ intake, so as to prevent Na^+ accumulation, water retention and high blood pressure. In mammalian cells, the intracellular accumulation of Na^+ and its active transport is controlled by the Na,K-ATPase, a heterodimer composed of an α subunit with catalytic activity, and a β subunit, which is a chaperone. In kidney tubule epithelial cells, Na,K-ATPase plays a central role in Na^+ reabsorption, establishing the Na^+ gradients required for Na^+ uptake by apical transporters, while at the same time being responsible for much of the Na^+ extrusion from the basolateral surface, so that Na^+ can re-enter the blood.

Na,K-ATPase also plays a major role in regulating intracellular Na^+ levels, and in these regards is at the center of a number of cellular signaling pathways which respond to changes in intracellular Na^+ [1]. The signaling pathways generated by G Protein Coupled Receptors (GPCRs) activated by the renal effectors produced in response to changes in extracellular Na^+ have been extensively studied. However, the interrelationships between the GPCR generated signaling that occurs in response to changes in extracellular Na^+ , and the signaling networks generated in response to changes in intracellular Na^+ are not clearly understood.

Included amongst these signaling pathways is a network that centers around salt-inducible kinase 1 (SIK1), and that responds to changes in intracellular Na^+ (and subsequent calcium-related signals) [1]. In the adrenal, where SIK1 was discovered [2], SIK1 represses cAMP dependent transcription through its ability to phosphorylate cAMP-Regulated Transcriptional Co-activators (CRTCs), or Transducers of Regulated CREB (TORCs) [3–5]. Following the phosphorylation of CRTCs by SIK1, CRTCs are sequestered in the cytoplasm, where they interact with 14-3-3 proteins [4]. Following exposure to agents that increase cAMP, SIK1 is phosphorylated, and inactivated. However, this is not sufficient to activate CRTCs. An increase intracellular Ca^{2+} ($\text{Ca}^{2+}_{\text{in}}$) must occur to activate Calcineurin (Protein Phosphatase 2B). Activated Calcineurin dephosphorylates CRTCs, which translocate to the nucleus, and binding to CREB in a subset of promoters.

One of these promoters is the promoter of the *atp1b1* gene, which encodes for the Na,K-ATPase β subunit [6]. Genetic regulation of the Na,K-ATPase β subunit is of particular importance, because the β subunit is limiting for heterodimer formation in many cell types [7]. Thus, increases in the level of the β subunit result in an increase in heterodimer formation, and thus in an overall increase in the level of Na,K-ATPase, and may occur in response to a number of stimuli.

A number of renal effectors are produced by the kidney following changes in Na^+ intake, including effectors that elicit either natriuretic or anti-natriuretic responses [8]. One of these effectors, norepinephrine, stimulates Na^+ reabsorption when dietary Na^+ declines, and

another, dopamine, inhibits Na^+ reabsorption when dietary Na^+ increases. Yet another renal effector, Prostaglandin E_2 (PGE_2), can either stimulate or inhibit Na^+ reabsorption, depending upon the nephron segment, and signaling pathways involved. All of these renal effectors have been observed to regulate Na^+ reabsorption in the renal proximal tubule (RPT) via their effects on Na,K-ATPase . The renal proximal tubule (RPT) is particularly important, because it reabsorbs more than 80% of the filtered Na^+ , and much of this reabsorption occurs via Na,K-ATPase [9].

Investigations concerning the regulation of Na,K-ATPase by natriuretic factors including dopamine, and anti-natriuretic factors, including norepinephrine, have been concerned almost exclusively with events occurring at the membrane level, including the insertion/removal of pre-existing transporters in the basolateral membrane [10, 11]. These natriuretic factors and anti-natriuretic factors have been found to act via common signal transduction pathways which are subject to bidirectional control via phosphorylation and dephosphorylation events [12].

Phosphorylation/dephosphorylation events are also a rapid means to control gene expression in response to extracellular signals [4]. However, the changes in gene expression that occur in response to renal effectors such as dopamine and norepinephrine are poorly understood. Both norepinephrine and dopamine can cause increases in intracellular cAMP that result in the activation of Protein Kinase A (PKA). PKA has been observed to directly phosphorylate the cAMP responsive element (CRE) binding protein (CREB) at Ser133, resulting in the interaction of CREB with CREB binding protein (CBP), which results in an increase in the rate of initiation of transcription by RNA polymerase II [13]. However, CREB phosphorylation by PKA at Ser133 (and the subsequent interaction of CREB with CBP) in and by itself only activates a small proportion of CREB phosphorylated genes (which differ with regards to cell type) [14].

The activation of a number of genes in response to cAMP and Ca^{2+} signaling requires cAMP-Regulated Transcriptional Co-activators (CRTCs), or Transducers of Regulated CREB (TORCs), in addition to CBP [3–5]. CRTCs interact with the bZIP domain of CREB, in a manner that causes an increase CREB activity [3, 5]. The association of CRTCs and CBP with CREB is not mutually self-exclusive. CRTCs interact with the bZIP domain, unlike CBP, which interacts with the KID domain [4]. Although the phosphorylation of CREB is not required for CRTCs and CREB to interact, both phosphorylation and dephosphorylation events must nevertheless occur, albeit at another level.

Previously, we presented evidence that the activity of the Na,K-ATPase in MDCK cells and primary RPT cells is subject to regulation at the transcriptional level by PGE_1 and PGE_2 . We observed that PGE_1 causes a 10-fold increase in β subunit mRNA in MDCK cells, while the increase in α subunit mRNA was minimal [15]. For this reason β subunit regulation was examined in detail [6, 15–19]. PGE_1 and PGE_2 stimulate transcription of *atp1b1* in primary RPT cells and MDCK cells, and evidence has been obtained that CREB was involved [6, 20, 21]. We found that CREB associates with 2 Prostaglandin Regulatory Elements (PGRE1 and PGRE3) on the *atp1b1* promoter [6, 22]. Treatment of primary RPT cells and MDCK cells with either PGE_1 or PGE_2 , results in the phosphorylation of CREB on the *atp1b1* promoter,

such that CREB with CBP interact [6, 15]. In this report, evidence is presented that in primary RPT cells, CRTCs are involved in the regulation of the Na,K-ATPase *atp1b1* gene in response to PGE₁, PGE₂, norepinephrine and dopamine. The involvement of SIK1 is also examined.

2. Materials and Methods

2.1. Materials

Hormones, human transferrin and other chemicals were from Sigma Aldrich Chemical Corp. (St. Louis, MO). Collagenase IV was from Worthington (Freehold, NJ). Dulbecco's Modified Eagle's Medium (DME), Ham's F12 Medium (F12), soybean trypsin inhibitor, lipofectamine, the Galacto-Star β -Galactosidase Assay System, and the Fluo-4 Direct Assay Kit were from Life Technologies Corp. (Carlsbad, CA). Prism 6 software was from GraphPad, Inc. (San Diego, CA). The cAMP Select EIA Kit was from Cayman Chemical Company (Ann Arbor, MI), and the BCA Protein Assay Kit was from Thermo Scientific (Rockford, IL). The pSV β gal plasmid and the reporter lysis buffer were from Promega (Madison, Wisc). The plasmid pH β 1-1141Luc was from Dr. Jerry Lingrel (Univ. of Cincinnati, Cincinnati, Ohio)[23]. The SIK1-K56M expression vector and the empty vector, pcDNA3 was from Hiroshi Takemori (National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan) [24]. The CRTC1, CRTC2, and CRTC3 vectors, as well as the empty vector pcDNA 3.1/V5/HisA were from Dr. Dong-Yan Jin, Dept of Biochemistry of The University of Hong Kong [25]. WT CREB (pRc/RSV-Flag-CREB), pRc/RSV-Flag-CREB R314, dominant negative TORC1 (pcDNA3-TORC1₁₋₁₄₇-EGFP), and the empty vector pcDNA3-EGFP, were from Jean-Rene Cardinaux, University of Lausanne, Lausanne, Switzerland [26]. The rabbit anti-pan CRTC (1–42) antibody was obtained from EMD Millipore, Billerica, Mass., and the anti-CRTC1 antibody was from Santa Cruz Biotechnology, Santa Cruz, Calif.

2.2. Primary RPT Cell Cultures

Primary rabbit RPT cell cultures were initiated as described previously [27]. To summarize, male New Zealand White rabbits (2–2.5 kg) were euthanized following a procedure approved by the IACUC of the University at Buffalo. The kidneys were perfused via the renal artery, first with Phosphate Buffered Saline (PBS) and then sterile 0.5% (w/v) iron oxide. The renal cortex was sliced, and slices disrupted using a sterile Dounce homogenizer (loose pestle). After separating tubule fragments through 253 μ and 83 μ mesh nylon sieves, proximal tubules and glomeruli on the 83 μ sieve were transferred into a 50 ml conical tube containing basal medium (a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F12 (DME/F12), pH 7.4 with 15 mM HEPES, 20 mM Na⁺ bicarbonate, penicillin (92 units/ml and 0.01% kanamycin). The glomeruli (with iron oxide) were removed using a sterile stir bar. The proximal tubules were treated with 0.05 mg/ml collagenase class IV and 0.5 mg/ml soybean trypsin inhibitor (2 min; 23° C), washed twice by centrifugation at 500 $\times$ g, re-suspended in basal medium, and plated into 35 mm cultures dishes containing basal medium supplemented with 5 μ g/ml bovine insulin, 5 μ g/ml human transferrin, 5 $\times$ 10⁻⁸ M hydrocortisone, and 10⁻⁶ M indomethacin (Medium RK-2).

Indomethacin was added to inhibit prostaglandin biosynthesis. The medium was changed the day after plating, and every other day thereafter.

2.3. Transient Transfection Studies

Primary cultures were cotransfected with pH β 1-1141 Luc, a human β 1 subunit promoter/luciferase construct (1 μ g), and pSV β gal (0.2 μ g) utilizing lipofectamine, as previously described [21]. The next day, the cultures were incubated for 2 hrs in fresh Medium RK-2 in a 5% CO₂/95% air humidified environment. Appropriate effectors were added, followed by a 4 hr incubation. The monolayers were then solubilized in Reporter Lysis Buffer, centrifuged 1 minute at 13,600 $\times$ g at 4° C, and assayed for luciferase activity, in luciferase assay buffer (20 mM Tricine, 1.07 mM (MgCO₃). 4Mg(OH)₂, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μ M Coenzyme A, 470 μ M luciferin and 530 μ M ATP). Emitted light was measured in a BioTek Plate Reader [21].

Luciferase determinations were individually normalized with respect to β -gal activity (determined using the Galacto-Star System). Average luciferase values were calculated from quadruplicate determinations ($\pm$ the Standard Error of the Mean (SEM)), and compared to the Control value (in untreated cultures). The statistical significance of stimulatory and inhibitory effects was assessed by means of a One Way analysis of variance (ANOVA), and the Newman-Keuls Multiple Comparison Test (using Prism 6 software). Differences were significant when $p < 0.05$. The effects of regulatory molecules were determined in at least 3 independent culture sets.

2.4. Western Analysis

Samples were equalized with regards to protein by the Bradford method (7), separated by electrophoresis through 7.5% SDS/polyacrylamide gels, and transferred to nitrocellulose as previously described [28]. Blots were blocked 1 h in Tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween 20 (TTBS), and incubated 2hr with primary antibody in TTBS, followed by 6 washes in TTBS (10 min/wash). After 45-min with a horseradish peroxidase (HRP) conjugated-secondary antibody, blots were washed seven times with TTBS (15 min/wash), followed by an incubation with Immun-Star HRP Luminol/Enhancer (Bio-Rad). Bands were visualized using LucentBlue X-ray film.

2.5. cAMP Determinations

Primary RPT cells were grown in 48 well plates in Medium RK-1. Two hr prior to treatment with agonists, the medium was changed, and the cultures were incubated at 37°C in a humidified 5% CO₂/95% air environment. Subsequently, agonists were added to the medium, and the incubation continued at 37°C in a humidified 5% CO₂/95% air environment. At the end of the incubation period, the medium was removed, and assayed directly for cAMP using a cAMP Select EIA Kit. In order to measure intracellular cAMP, 1 N HCl was added into each well after removing the medium. The cells were then removed from the dish using a rubber policeman, and the resulting cell suspension was centrifuged at 1,000 $\times$ g for 10 min. The cAMP associated with the cell lysates were assayed using a cAMP Select EIA Kit. For each culture condition, assays were conducted using 3 independent samples, each of which was assayed in triplicate. Aliquots of each sample were

placed into the individual wells of a 96 well plate, and incubated with cAMP antiserum in the presence of a cAMP tracer (acetylcholinesterase (AChE) linked to cAMP). At the end of the incubation, the quantity of cAMP tracer bound to the cAMP antiserum was determined using Ellman's Reagent, which contains the substrate to AChE. The product of the enzymatic reaction generated by Ellman's reagent and AChE has a distinct yellow color, measured in a BioTek Plate Reader at 412 nm. The proportion of the cAMP tracer that binds to the antibody during the incubation is inversely proportional to the amount of free cAMP present in the well. The amount of cAMP tracer bound to each well was corrected for nonspecific binding to obtain a binding value, B. The maximum binding value, B_0 , was determined in wells incubated in the absence of cAMP. B/B_0 values were calculated for each sample, including cAMP standards. Using the results obtained from the cAMP standards, a linear plot of $\text{logit}(B/B_0)$ vs. $\text{log}(\text{cAMP concentration})$ was constructed, where $\text{logit}(B/B_0) = \ln \{B/B_0/(1-B/B_0)\}$. The slope and intercept of the best-fit line were obtained by means of linear regression analysis, using GraphPad Prism 6 software. By this means, the cAMP concentration in each of the cell lysates was determined, and standardized with regards to protein (determined using the BCA protein assay). The final cAMP values were averages of triplicate determinations $\pm$ SEM. The statistical significance of differences between treated and untreated cultures was evaluated by determining whether $p < 0.05$. P values were determined using the 2 tailed t test, corrected for multiple comparisons using the Holm-Sidak method.

Ca²⁺_{in} Assay—In order to determine the effect of individual agonists on Ca²⁺_{in} levels, primary RPT cells were grown in 96 well plates in Medium RK-1. The medium was changed to Medium RK-1 buffered with HEPES, rather than sodium bicarbonate, 2 hrs prior to the assay and the cultures were incubated at 37° C. One hr prior to the assay, 2x Fluo-4 Direct calcium reagent loading solution was added into individual wells. The calcium reagent loading solution contains Fluo-4-AM as well as 5 mM probenidol, added to prevent efflux of the dye. The plate was incubated for 30 min at 37°C and 30 min at 23° C. Subsequently, appropriate effectors were added to individual wells. The fluorescence emission at 516 nm was measured at 23° C in a BioTek plate reader following excitation at 494 nm. The concentration of cytosolic Ca²⁺ was determined using the equation $[Ca^{2+}] = K_d (F - F_{min}) / (F_{max} - F)$ [29], where F_{max} is the fluorescence signal after the addition of the Ca²⁺ ionophore ionomycin (10 μ M) (at the end of an experiment), F_{min} is the fluorescence signal obtained after the subsequent addition of MnCl₂, and the K_d is 345 nM [30]. A similar F_{min} value was obtained following an incubation with 2 mM EGTA and 10 μ M ionomycin [31, 32]. F values were corrected for background fluorescence (F_{bkg}), determined following the addition of 2 mM EGTA and 1% Triton X100.

3. Results

3.1. Expression of CRTCs in Primary RPT Cells

Previously, we presented evidence that CREB is involved in transcriptional regulation of the Na,K-ATPase β subunit gene *atp1b1* in cultured kidney tubule epithelial cells [6, 17, 19]. In order to determine initially whether CRTCs contribute to transcriptional regulation of *atp1b1*, the expression of endogenous CRTCs in primary rabbit RPT cells was examined by

Western analysis using a pan-CRTC antibody. The results, shown in Fig. 1A, indicate that a) the major 67 kd band had the same molecular weight as the band observed on a CRTC1 blot of primary rabbit RPT cells (Fig. 1B), and thus that b) rabbit CRTC1 is the major CRTC isoform expressed in primary RPT cells.

3.2. Effect of CRTC Expression Vectors on *atp1b1* Transcription

In order to further determine whether CRTCs are involved in regulating transcription in RPT cells, primary RPT cells were cotransfected with the Na,K-ATPase $\beta 1$ promoter Luciferase construct pH $\beta 1$ -1141Luc, and individual CRTC expression vectors, encoding for each of the 3 CRTC isoforms, CRTC1, CRTC2, and CRTC3, respectively. The cultures were incubated 4 hrs in the presence or absence of PGE₁. Subsequently, luciferase assays were conducted. Fig. 2A shows that CRTC1, CRTC2 and CRTC3 all significantly increased transcription in the absence of PGE₁, as compared with untreated control cultures, cotransfected with the pcDNA3.1, the empty vector (EV). The greatest stimulation that was obtained in the absence of PGE₁ was observed with CRTC3. A significant PGE₂ stimulation was observed in the presence of each of the CRTCs (vs. untreated level for the same CRTC isoform). Thus, these results indicate that CRTCs stimulate *atp1b1* transcription both in the presence, as well as in the absence of PGE₁.

Previously, we presented evidence indicating that the PGE₁ stimulation of *atp1b1* transcription depends the presence of a CREB binding site (PGRE3), as well as an adjacent Sp1 site in the *atp1b1* promoter [6]. The requirement for both sites could be explained by a CREB/Sp1 interaction [6]. In order to determine whether the stimulatory effect of CRTCs was similarly dependent upon a CREB binding site and an adjacent Sp1 site, transient transfection studies were conducting using pLuc MCS $\beta 72$ -164, a construct containing PGRE3 (CAGTCCCTGC), and an adjacent Sp1 site (i.e. a GC box) [6]. In addition, the CRTC3 expression vector and the corresponding EV were employed.

Fig. 2B shows that PGE₁ increased transcription 30 fold in primary RPT cells cotransfected with the PGRE3 construct and pcDNA3.1. In primary cultures cotransfected with CRTC3 as well as PGRE3, the rate of basal transcription increased 12 fold (as compared with untreated cultures with the EV). A further increase in transcription was observed when the primary RPT cell cultures (which had been cotransfected with PGRE3 and CRTC3) were treated with PGE₁. This latter PGE₁ stimulation was significantly greater than the PGE₁ stimulation in cultures cotransfected with PGRE3 and the EV. Thus, these results indicate that CRTC3 increased both the PGE₁ stimulation, as well as basal transcription in the construct containing PGRE3.

However, (as shown in Fig. 2B) the stimulatory effect of CRTC3 was lost when cotransfecting with a mutant construct (PGRE3 GC trans) in which the Sp1 site adjacent to PGRE3 was translocated further downstream. Not only did the rate of basal transcription decline with the PGRE3 GC mutant, but, in addition, the PGE₁ stimulation was completely lost. These results indicate that the stimulatory effect of CRTC3 is dependent upon Sp1 as well as CREB.

In order to examine the requirement for endogenous CRTCs, a Dominant Negative (DN) form of CRTC was employed, which consists of the first 147 amino acids of CRTC1, with the nuclear localization signal and the CREB binding domain fused to EGFP [26]. The cultures were cotransfected with the PGRE3 construct, and either the DN CRTC expression vector, or the EV (pEGFP). Subsequently, a portion of the cultures was treated with PGE₁. Fig. 2 C shows that the PGE₁ stimulation significantly declined in the presence of the DN CRTC. Thus, these results indicate that the PGE₁ stimulation depends upon endogenous CRTCs.

3.3. Dependence of *atp1b1* Transcription on Endogenous CRTCs

The effect of the DN CRTC expression vector on the stimulatory effect of PGE₂ was also examined, because PGE₂ is the PGE isoform produced in the kidney. In this study, primary RPT cells were cotransfected with pHβ1-1141Luc and the DN CRTC expression vector (or the EV), followed by a 4 hr incubation with PGE₂. Fig 3A shows that PGE₂ increased transcription 6 fold in primary RPT cells cotransfected with the EV (pEGFP). In contrast, the PGE₂ stimulation was completely lost in cultures cotransfected with the DN CRTC. In order to study the involvement of endogenous CRTCs further, transient transfection studies were conducted with the CREB R314A expression vector. The arginine 314 to alanine mutation in CREB prevents CRTC binding to the bZIP domain of CREB in a dominant negative manner [26]. Figure 3B shows that when primary RPT cells are cotransfected with pHβ1-1141Luc and CREB R314A the PGE₂ stimulation not observed (unlike the PGE₂ stimulation observed in primary RPT cells cotransfected with WT CREB).

3.4. Involvement of CRTCs in Regulation of Na,K-ATPase by Norepinephrine

In order to determine whether CRTCs play a more generalized role in transport regulation in the RPT, the effects of norepinephrine and dopamine were examined. Renal nerves produce norepinephrine when dietary Na⁺ levels increase, stimulating Na,K-ATPase activity in the RPT, and thereby increasing Na⁺ reabsorption. Our previous transient transfection studies indicated that norepinephrine stimulates *atp1b1* transcription in RPT cells, in addition to its previously studied effects at the membrane level [19].

In order to examine the involvement of CRTC1 in transcriptional regulation by norepinephrine, the effects of the DN CRTC and CREB-R314A vectors on transcription control by norepinephrine were studied. Fig. 4A shows a stimulatory effect of norepinephrine in primary RPT cells cotransfected with the empty EGFP vector. The norepinephrine stimulation was completely lost in cultures cotransfected with the DN CRTC. The effect of CREB R314A was also examined, as shown in Fig. 4B. While norepinephrine had a significant stimulatory effect on transcription (3.7 ± 0.3 fold) in primary RPT cells cotransfected with WT CREB, the stimulation was significantly diminished by the CREB R314A mutant.

In order to determine whether the norepinephrine stimulation is receptor-mediated, the effect of adrenergic antagonists on the stimulatory effect of norepinephrine was studied. Fig 4C shows that both α1 and α2 antagonists, prazosin and yohimbine, respectively, inhibited the norepinephrine stimulation by over 80%, unlike propranolol, a β antagonist. These results

indicate that the norepinephrine stimulation is mediated by $\alpha 1$ and $\alpha 2$ adrenergic receptors. Fig. 4D shows that similarly, the $\alpha 1$ and $\alpha 2$ agonists, phenylephrine and guanabenz, respectively, stimulated transcription. However, in addition the $\beta 1$ agonist albuterol and $\beta 2$ agonist metoprolol was significantly stimulatory. These latter observations suggest that β receptor activation can also increase transcription, although this does not necessarily occur in response to norepinephrine.

3.5. Involvement cAMP and Ca^{2+}

The results obtained above indicate that the stimulatory effect of norepinephrine, on *atp1b1* transcription is dependent upon CRTCs, and that Gq-coupled α and Gs-coupled β adrenergic receptors are involved. Our previous studies similarly indicate that Gq-coupled EP1, as well as Gs-coupled EP2 and EP4 receptors are involved in mediating the PGE_1 and PGE_2 response [21]. In order to determine whether the stimulatory effects of norepinephrine, PGE_1 and PGE_2 on *atp1b1* transcription in primary RPT cells are indeed dependent upon cAMP and Ca^{2+} signaling, the effects of these agonists on intracellular cAMP and Ca^{2+} levels were examined.

Fig. 5A shows that norepinephrine increased intracellular cAMP 7 fold after a 5 min incubation. PGE_1 and PGE_2 , also significantly increased intracellular cAMP, albeit to a lower extent. In contrast, as shown in Fig. 5B, PGE_1 and PGE_2 stimulated extracellular cAMP production to a greater extent than norepinephrine following a 6 hr incubation (Fig. 5B). The effect of PGE_1 , PGE_2 , and norepinephrine on $\text{Ca}^{2+}_{\text{in}}$ levels was examined using Fluo4 (as illustrated in Fig. 6A, B, and C). The basal level of $\text{Ca}^{2+}_{\text{in}}$ increased immediately (within 20 sec) following the addition of each of these agonists. After 20 min, the $\text{Ca}^{2+}_{\text{in}}$ level had increased by 1.9 fold in PGE_1 treated cultures (Fig. 6A), 1.6 fold in PGE_2 treated cultures (Fig. 6B), and 1.7 fold in norepinephrine treated primary RPT cell cultures (Fig. 6C).

The effect of the PKA inhibitor H89, and the Ca^{2+} /Calmodulin-dependent kinase type II inhibitor (CaMK II) KN62 on the norepinephrine and PGE_2 response was also examined. Fig. 7A and 7B show that H89 and KN62, respectively, completely inhibited the stimulatory effects of norepinephrine, and PGE_2 . Thus, these results are consistent with hypothesis that the stimulatory effects of norepinephrine and PGE_2 on *atp1b1* transcription depend upon both cAMP and Ca^{2+} signaling. This is a hallmark of CRTC-activated transcription [33].

3.4. Role of CRTCs in Regulation by Dopamine

Previous studies indicate that dopamine inhibits Na,K-ATPase in the RPT, resulting in natriuresis [8]. Acute inhibition of Na,K-ATPase by dopamine is associated with increased endocytosis of Na,K-ATPase, and a decline in the number of Na,K-ATPases in the basolateral membrane [11, 34]. This effect of dopamine depends upon an increase in intracellular Na^+ (Na^+_{in}) (which is a consequence of the increase in luminal Na^+ that occurs during a high salt diet). The Na^+ ionophore monensin causes such an increase in Na^+_{in} in RPT cells in vitro, and for this reason has been employed by investigators studying the acute inhibitory effects of dopamine on Na,K-ATPase in cultured RPT cells [35]. An increase in intracellular Ca^{2+} is a result of the monensin-induced increase in Na^+_{in} in RPT cells, and is

essential for the acute inhibitory effect of dopamine on Na,K-ATPase. Such an increase in intracellular Ca^{2+} also occurs in primary RPT cells treated with monensin (Fig. 6D). For these reasons, we determined whether monensin similarly affects transcriptional regulation of Na,K-ATPase by dopamine, and whether CRTCs are involved.

Towards these ends, primary RPT cells were transiently transfected with pH β 1-1141Luc, and then incubated 4 hrs with dopamine and/or monensin. Fig. 8A shows that both 10 μM dopamine and 5 μM monensin were stimulatory. However, when the cultures were incubated with 10 μM dopamine, as well as 5 μM monensin, transcription decreased by 80% (relative to monensin alone), such that the monensin stimulation was completely lost. The effect of L-dopa was also examined (Fig. 8A), because L-dopa is the precursor utilized in the synthesis of dopamine by aromatic L-amino acid decarboxylase in the RPT [36]. A stimulation was observed when 10 μM L-dopa was added alone (similar to that of dopamine), and L-dopa completely inhibited the stimulatory effect of monensin (also similar to the effect of dopamine). Thus, the observed effects of L-dopa can be explained by its metabolism to dopamine.

In order to determine whether transcriptional regulation by dopamine depends upon PGRE3, and whether CRTCs are involved, a) transient transfection studies were conducted with either the PGRE3 vector, or the PGRE3 GC trans mutant (Fig. 8B), and in addition b) transient transfection studies were conducted with pH β 1-1141 Luc and the DN CRTC (Fig. 8C). Fig. 8B shows that in primary RPT cells transfected with the PGRE3 vector, that a) monensin and dopamine were stimulatory when added individually, and b) dopamine inhibited the monensin stimulation. However, these effects of dopamine and monensin were not observed in primary RPT cells transfected with the PGRE GC trans mutant. Similarly, as shown in Fig. 8C, the effects of dopamine and monensin were completely lost in primary RPT cells cotransfected with the DN CRTC. Thus, these results are consistent with the hypothesis that the effects of monensin and dopamine are indeed dependent upon CRTCs, and their association with TFs bound to PGREs and adjacent Sp1 sites.

In order to determine whether dopamine acts via either DA1 or DA2 receptors, the effects of DA1 and DA2 agonists and antagonists were examined. As shown in Fig. 8D, the DA1 antagonist SCH23390 completely prevented the increase in transcription that was observed in the presence of dopamine. Only a partial inhibition of transcription was obtained in the presence of the DA2 antagonist L741, 626. Consistent with these observations are the stimulatory effects the DA1 agonist SKF 38,393 and the DA2 agonist quinpirole shown in Fig. 8E. Thus, these results indicate that the DA1 receptor is primarily involved in the dopamine stimulation, although DA2 receptor agonists can also elicit effects.

3.5. Role of Salt Inducible Kinase 1 (SIK1) in Transcriptional Regulation

SIK1 was also studied because, 1) SIK1 has been shown to play a role in the acute regulation of Na,K-ATPase in the RPT [37], and 2) SIK1 can directly phosphorylate CRTCs [38–41], causing CRTCs to localize to the cytoplasm, thus preventing their nuclear effects. When SIK1 is phosphorylated, and inactivated by PKA, CRTCs can be dephosphorylated by calcineurin, and translocate to the nucleus, where they interact with CREB. Thus, the

hypothesis was examined that SIK1 does indeed limit transcription of *atp1b1* in RPT cells, in a manner that can be altered by local renal effectors.

In order to examine this hypothesis initially, the effects of kinase defective SIK1-K56M [42]) on transcriptional regulation by PGE₁ and norepinephrine were examined. Fig 9A shows that basal transcription increased in primary RPT cells cotransfected with SIK1-K56M and pHβ1-1141Luc (vs. cultures cotransfected with the empty vector (EV)). Transcription similarly increased in norepinephrine, and PGE₁ treated primary RPT cells cotransfected with pHβ1-1141Luc and SIK1-K56M, although the level of transcription was not significantly higher than in untreated cultures cotransfected with SIK1-K56M. In contrast, WT-SIK had no significant affect on the level of transcription (Taub, unpublished).

These observations are consistent with the hypothesis that in untransfected cells, SIK1 is phosphorylated and inactivated following treatment with either norepinephrine or PGE₁, with a consequent increase in nuclear CRTCs, and transcription. In this case, norepinephrine and PGE₁ would no longer be required to elicit these effects to increase in *atp1b1* transcription in the presence of SIK1-K56M.

The effects of SIK1-K56M, on transcriptional regulation by dopamine and monensin were also studied. As shown in Fig. 9B, dopamine added alone was stimulatory in cultures cotransfected with the EV. In primary RPT cell cultures transfected with SIK1-K56M, the rate of transcription observed with dopamine was similarly elevated relative to untreated EV controls, yet did not differ significantly from untreated SIK1-K56M cotransfected cultures. Thus, the stimulatory effect of dopamine can be explained at least in part, by the inactivation of SIK1 (presumably by PKA), with the consequent nuclear interaction of CRTC with CREB.

Figure 9B also shows that monensin significantly reduced transcription in SIK1-K56M cotransfected cultures. While dopamine reduced transcription in the presence of monensin (in cultures cotransfected with the EV), dopamine did not have a significant effect in the presence of monensin in SIK1-K56M cotransfected cultures. These results can be explained, if both the stimulatory effect of monensin, and the inhibitory effect of dopamine depend upon SIK1.

4. Discussion

In this report, evidence is presented that CRTCs and SIK1 are both involved in transcriptional regulation of the RPT Na,K-ATPase β1 subunit gene, *atp1b1* in response to PGE₂, norepinephrine, dopamine, and increased intracellular Na⁺. The involvement of CRTC1 in mediating the effects of PGE₁ and PGE₂ in particular is supported by the observation that the PGE₂ stimulation is inhibited by an expression vector encoding for a DN-CRTC (a CRTC1 deletion mutant, containing only the CREB binding domain and the nuclear localization domain), as well as by CREB R314A (full length CREB with a mutation in the CRTC binding site). Presumably, the stimulatory effect of CRTCs on transcription is limited by the phosphorylation of CRTCs by SIK1 (preventing their nuclear translocation). In this case, PGE₁ and PGE₂ would increase *atp1b1* transcription at least in part by phosphorylating and inactivating SIK1. Consistent with this hypothesis, a kinase dead SIK1

mutant (SIK-K56M), 1) caused an increase in the rate of *atp1b1* transcription that was equivalent to that obtained with PGE₁, and 2) transcription did not increase any further when SIK-K56M transfected cells were treated with PGE₁. Similar evidence was obtained supporting the involvement of CRTCs and SIK1 in mediating the effects of norepinephrine and dopamine on transcription.

The involvement of CRTCs in transcriptional regulation of *atp1b1* is consistent with our previous studies of transcription control of *atp1b1* in MDCK cells and primary RPT cells [19, 43]. In these previous studies, Prostaglandin Regulatory Elements (PGREs) were identified in the *atp1b1* promoter, which are required for the stimulatory effects of PGE₁ and PGE₂. Our previous studies indicated that PGRE1 and PGRE3 serve as binding sites for CREB [22, 43]. When CREB binds to either PGRE1 or PGRE3, CREB also associates with Sp1, located on an adjacent Sp1 site. Although the CREB/Sp1 association is essential for the PGE₁ and PGE₂ stimulation, the PGE₁ and PGE₂ stimulation nevertheless also requires the phosphorylation of CREB, and the association of CREB with CBP. This report indicates that in addition, CREB activation in the *atp1b1* promoter depends upon CRTCs [3, 44].

CRTCs are recently discovered transcriptional coactivators that increase transcriptional activation of CREB, on a subset of target genes subject to control by cAMP and Ca²⁺. The CRTC family consists of 3 members, including CRTC1, CRTC2 and CRTC3. In primary RPT cells, all 3 CRTC expression vectors, CRTC1, CRTC2 and CRTC3, stimulated *atp1b1* transcription. Although the involvement of all 3 endogenous CRTCs cannot be excluded, our Western analysis indicates that CRTC1 is the major CRTC isoform expressed in RPT cells.

Although previous reports of CRTC function in the kidney are lacking, CRTCs have been shown to have functional roles in other tissues. In the brain CRTC1 stimulates the transcription of the gene encoding for Brain Derived Neurotrophic Factor (BDNF), and produces a long-lasting increase in synaptic transmission in the hippocampus [26, 45, 46]. Similar mechanisms occur in the CNS, which express high levels of CRTC1 [45, 47]. In arcuate neurons in the hypothalamus, the expression of cocaine and amphetamine-regulated transcript prepropeptide (Cartpt) and Kiss1 increases following the interaction of CRTC1 with CREB. While Cartpt inhibits food intake in response to leptin, Kiss1 promotes fertility in response to leptin, by stimulating secretion of hypothalamic Gonadotropin-Releasing Hormone (GnRH), and subsequently, luteinizing hormone (LH) [48].

The other CRTC isoforms are important in insulin-sensitive tissues, where they control a number of processes. In the liver, glucagon causes CRTC2 to become dephosphorylated during fasting because SIK2 (which normally phosphorylates CRTC2), is inhibited due to its phosphorylation by PKA. CRTC2 dephosphorylation, results in increased expression of gluconeogenic genes, including Phosphoenolpyruvate Carboxykinase (PEPCK), and glucose 6-phosphatase [4]. In contrast, during feeding, glucose activates CRTC2 signaling in pancreatic islets, stimulating insulin production [4]. CRTC3 also regulates gene expression in other insulin sensitive tissues, including adipose tissue, where it attenuates catecholamine signaling [4], and skeletal muscle, where it stimulates the remodeling of myofibers [49].

Our previous studies have indicated that transcriptional regulation of *atp1b1* by PGE₁ and PGE₂ shares an important feature distinctive of the pathways controlling CRTCS, i.e. that both the cAMP and Ca²⁺ pathways must be activated in order for PGE₁ and PGE₂ to exert their control at the transcriptional level [6]. The requirement for cAMP and Ca²⁺ can be explained by their ability to control the state of phosphorylation of CRTCs, and hence the nuclear localization of CRTCs. Under resting conditions, when these pathways are not active, CRTCs are phosphorylated by SIK, and are sequestered in the cytoplasm, forming complexes with 14-3-3 proteins. When both the cAMP and Ca²⁺ pathways activated, SIK1 is phosphorylated (by PKA) and inactivated, while Protein Phosphatase 2B (Calcineurin) is phosphorylated and activated as a consequence of the binding of Ca²⁺ and calmodulin. As a consequence, CRTCs are dephosphorylated, translocate to the nucleus and interact with CREB on appropriate target genes.

In primary RPT cells, the effects of CRTCs on *atp1b1* transcription are presumably limited when CRTCs are phosphorylated by SIK1 (preventing the nuclear localization of CRTCs). In this case, PGE₁ and PGE₂ would increase *atp1b1* transcription at least in part by stimulating the phosphorylation and inactivation of SIK1. Consistent with this hypothesis, the kinase dead SIK1 mutant (SIK1-K56M), caused the rate of basal *atp1b1* transcription to increase to the same extent as the increase caused by PGE₁ (in primary RPT cells transfected with the empty vector). No further increase in transcription was observed when SIK1-K56M transfected cells were treated with PGE₁. Thus, the results with SIK1-K56M are consistent with the involvement of CRTCs, as well as SIK1, in mediating the stimulatory effects of PGE₁ and PGE₂ on *atp1b1* transcription. As stated above, the studies with the DN-CRTC vector are similarly consistent with the involvement of CRTCs in mediating the stimulatory effect of PGE₁ and PGE₂ on *atp1b1* transcription.

The observed stimulatory effects of PGE₂ (and PGE₁) on *atp1b1* transcription are also consistent with previous reports of stimulatory effects of PGE₂ on Na⁺ and fluid reabsorption by the renal proximal tubule [50]. Our previous results indicate that the stimulatory effects of PGE₁ and PGE₂ on primary RPT cells on *atp1b1* transcription are mediated by Gs-coupled EP2 and EP4 receptors (which result in the stimulation of adenylate cyclase (AC), as well as Gq-coupled EP1 receptors (which result in the stimulation of phospholipase C (PLC) [19]. The ultimate result is increased Na,K-ATPase activity [19].

Our studies similarly indicate that CRTCs and SIK1 play a role in mediating the observed effects of norepinephrine, and dopamine on *atp1b1* transcription in primary RPT cells. The observed stimulatory effects of norepinephrine, and the inhibitory effects of dopamine at the transcriptional level a) are consistent with their known physiologic effects, b) ultimately result in either an increase, or a decrease in Na,K-ATPase activity, in the case of norepinephrine and dopamine, respectively [51], and thus c) may very well be physiologically significant.

Indeed, in the kidney, norepinephrine is anti-natriuretic, stimulating Na⁺ reabsorption by the RPT. Previous studies indicate that α adrenergic receptors mediate the stimulatory effect of norepinephrine on Na,K-ATPase in RPT cells [52]. The results of our studies with α adrenergic agonists, as well as antagonists, indicate that α adrenergic receptors are similarly

involved in stimulating *atp1b1* transcription. When RPT cells are incubated with α adrenergic agonists, calcineurin is activated [52]. Once activated, calcineurin dephosphorylates specific target proteins, including CRTCs [53]. As a consequence, CRTCs enter the nucleus, where they interact with CREB. If β adrenergic receptors are also involved in transcriptional regulation of *atp1b1* (as suggested by our studies with β adrenergic agonists), increased transcription is also a consequence of increased cAMP, as well as the phosphorylation and inactivation of SIK1 by PKA (resulting in the translocation of CRTCs into the nucleus).

The results obtained with primary RPT cells cotransfected with pH β 1-1141Luc and the kinase dead SIK1 mutant, SIK1-K56M, are consistent with this latter hypothesis. Indeed, SIK1-K56M caused the basal level of *atp1b1* transcription to increase to the level obtained with norepinephrine, both in primary RPT cells cotransfected with the empty vector, as well as in primary RPT cells cotransfected with SIK1-K56M. In any case, these results suggest that SIK1 can limit transcriptional of the *atp1b1* gene. However, both the PKA mediated phosphorylation and inactivation of SIK1, and the calcineurin-mediated dephosphorylation of CRTCs are most likely required for the nuclear translocation of CRTCs, and their stimulation of transcription of the *atp1b1* gene.

Dopamine causes natriuresis in the kidney, inhibiting Na⁺ reabsorption by the RPT. The inhibitory effect of dopamine on Na⁺ reabsorption has been attributed to the activation of dopamine with Gs-coupled D1-like receptors in the RPT, including D1 and D3 receptors, resulting in the activation of adenylate cyclase (AC), and PKA [54]. Our previous results indicate that PGE₁ and PGE₂ similarly activate AC via EP2 and EP4 receptors, in addition to activating calcium-mediated pathways via EP1 receptors [19]. However, these observations do not necessarily indicate that PGE₁, PGE₂ and dopamine act via the same mechanism. Indeed inhibition of Na⁺ reabsorption by renal D1-like receptors appears to involve the activation of distinct isoforms of AC, including the AC V isoform, PGE₁, whose effects on cAMP accumulation appear to involve AC VII [55]. PGE₁ and PGE₂ may nevertheless influence the effects of dopamine by activating EP1 receptors, which reportedly interact with D1A receptors, and in this manner affect dopamine action [19][56].

In this report, we observed that when dopamine is added alone, it stimulates transcription, similar to the effects of dopamine in alveolar lung epithelial cells [57]. However, in the RPT *in vivo*, this stimulatory effect of dopamine may not be physiologically significant, because renal dopamine is primarily produced when Na⁺ levels increase (which results in increased intracellular Na⁺ in the RPT). When we simulated this condition *in vitro*, using the Na⁺ ionophore monensin, an inhibitory effect of dopamine on *atp1b1* transcription was observed. This latter observation is consistent with the studies of Efendiev et al. [35] which indicated that the acute inhibitory effect of dopamine on Na,K-ATPase in cultured RPT cells similarly depends upon an increase in intracellular Na⁺ (caused experimentally by monensin). The inhibitory effect of dopamine on *atp1b1* transcription was lost in primary RPT cells cotransfected with the dominant negative SIK1 mutant, suggesting that CRTCs are also involved.

Our results are consistent with a previously proposed hypothesis that dopamine and norepinephrine exert their effects in the RPT via a bidirectional pathway that controls the phosphorylation of Na,K-ATPase [12]. According to this hypothesis, dopamine initiates a series of signaling events that result in the phosphorylation of Na,K-ATPase, and a reduction in Na,K-ATPase activity [12]. In contrast, norepinephrine and Ang II were proposed to increase $\text{Ca}^{2+}_{\text{in}}$, ultimately resulting in the dephosphorylation, and activation of Na,K-ATPase [12]. The Ca^{2+} mediated effects of norepinephrine were proposed to involve the activation of Calcineurin, and the subsequent activation of Protein Phosphatase 2A, which directly dephosphorylates, and activates Na,K-ATPase [52].

A number of studies have also indicated that the phosphorylation of Na,K-ATPase affects the ability of Na,K-ATPase to be retained in the basolateral membrane of RPT cells [11]. Dopamine was observed to cause a reduction in the level of basolateral Na,K-ATPase, presumably as a consequence of the phosphorylation of the α subunit of Na,K-ATPase by Protein Kinase C (PKC) ζ , and endocytosis [11]. Alpha-adrenergic agonists counteracted these effects of dopamine, by preventing the recruitment of clathrin to the plasma membrane, rather than by preventing α subunit phosphorylation [58].

Biochemical and biophysical studies indicate that Na,K-ATPase is subject to control by Na^{+} itself [59]. Na,K-ATPase operates below its maximal capacity when intracellular Na^{+} is at a basal level, which can range from 5–13 mM [35]. However, in the RPT such increases in intracellular Na^{+} can also stimulate Na,K-ATPase by an increase in $\text{Ca}^{2+}_{\text{in}}$, which results in the activation of CaM Kinase, and ultimately in the activation of SIK1. As a consequence, Protein Phosphatase 2A is activated, which dephosphorylates, and activates Na,K-ATPases in the basolateral membrane [1][37].

The results presented in this report indicate that SIK1 plays yet another role in the regulation of Na,K-ATPase in the RPT, via the phosphorylation of CRTCs, which cause CRTCs to be sequestered in the cytoplasm, and in this manner placing transcriptional regulation of *atp1b1* on hold (at least until renal dopamine is produced), while permitting control to occur at membrane level.

In this report, we also observed that *atp1b1* transcription increased in the presence of monensin. However transcription in the presence of monensin did not increase further in the presence of a kinase dead SIK1 mutant, and in fact was below the level observed in untreated cultures cotransfected with the SIK1-K56M mutant. This observation can be interpreted as indicating SIK1 is responsible for initiating other transcriptional events (distinct from those elicited by CRTCs), and that these transcriptional events no longer occur in this condition. Indeed, the stimulatory effect of monensin on the transcription of Myocyte Enhancer Factor 2 (MEF2) in an atrial myocyte cell line involves the phosphorylation of Histone Deacetylase 5 (HDAC5) by SIK1 (and/or CaMK1) [60]. HDACs may similarly play a role in transcriptional regulation of the *atp1b1* gene. The inhibitory effect of dopamine on *atp1b1* transcription was similarly lost after cotransfecting with the dominant negative SIK1. Thus, the inhibitory effect of dopamine on *atp1b1* transcription may similarly directly depend upon HDAC phosphorylation by SIK1. Further studies are in progress to examine this hypothesis.

Conclusions

Our results support the hypothesis that renal effectors including norepinephrine and PGE₂ stimulate transcription of the Na,K-ATPase β subunit gene *atp1b1* in renal proximal tubule cells via CRTCs. These observations are particularly significant, because the Na,K-ATPase β subunit level is limiting in the formation of Na,K-ATPase heterodimers, and thus in cellular Na,K-ATPase levels. The inhibitory effect of dopamine on *atp1b1* transcription is observed when intracellular Na⁺ is increased (using monensin in vitro). The latter condition results in a CRTC dependent stimulation of *atp1b1* transcription. Previously, SIK1 was shown to regulate Na,K-ATPase in the RPT at the membrane level. In this report, evidence is presented that SIK1 also plays a role in regulating Na,K-ATPase gene expression in RPT cells. SIK1 phosphorylates CRTCs in a number of experimental systems, causing CRTCs to be sequestered in the cytoplasm. Our results support the hypothesis that 1) SIK1 similarly acts in RPT cells, so as to limit the level of CRTCs in the nucleus, causing a reduction in *atp1b1* gene expression, and 2) that these effects of SIK1 on CRTCs are prevented by renal effectors, including norepinephrine and PGE₂. However, the stimulatory effect of monensin (and intracellular Na⁺) on *atp1b1* transcription apparently depends upon other nuclear effects of SIK1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PKA	protein kinase A
CRTC	cAMP-Regulated Transcriptional Coactivator
SIK1	Salt Inducible Kinase 1
RPT	renal proximal tubule

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Highlights

Dopamine, norepinephrine and PGE₂ regulate renal Na,K-ATPase at the gene level.

This involves regulation of renal *atp1b1* transcription by CRTCs.

In the kidney, SIK1 limits control by CRTCs, in addition to affects on membranes.

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Figure 1.

Expression of Endogenous CRTCs in Primary RPT Cells. A. The relative expression of endogenous CRTC1, CRTC2 and CRTC3 was determined in cell lysates by Western analysis using a pan-CRTC antibody. B. The expression of endogenous CRTC1 was determined in cell lysates by Western analysis using an anti-CRTC1 antibody.

Figure 2.

Control of Transcription by Exogenous CRTCs. A. Primary RPT cells were cotransfected with pH β 1-1141Luc, pSV β gal and either the expression vector CRTC1, CRTC2, CRTC3 or the empty vector (EV) pcDNA3.1. The following day primary RPT cells were incubated 4 hrs in either the presence or the absence of 280 nM PGE₁. B. Primary RPT cells were cotransfected with a) pLuc MCS β 72-164 (i.e. the PGRE3 vector) and CRTC3 or the EV, or b) the PGRE3 GC Trans Mutant and CRTC3 or the EV. The following day, the cultures were incubated 4 hrs in either the presence or absence of 280 nM PGE₁. After the 4 hr incubation, luciferase and β galactosidase activity was determined in quadruplicate for each condition. C. Primary RPT cells were cotransfected with the PGRE3 vector and either pEGFP (the EV), or DN TORC1. The following day, primary RPT cells were incubated 4 hrs in either the presence or the absence of 100 nM PGE₁. In A,B, and C, values are

expressed relative to the Control Value (untreated EV) $\pm$ SEM; for untreated cotransfected cultures, * $p < 0.05$ relative to the untreated control cotransfected with pcDNA3.1; for PGE₁ treated cultures, # $p < 0.05$ relative to untreated cultures cotransfected with the same vectors; in part A, ‡ $p < 0.05$ relative to untreated CRTC1 cotransfected cultures; for PGE₁ treated DN CRTC1 cotransfected cultures, in part C, † $p < 0.05$ relative to PGE₁ treated cultures cotransfected with the EV (pEGFP).

Figure 3.

Dependence of Transcription upon Endogenous CRTC1. A. Primary RPT cells were cotransfected with pH β 1-1141Luc and either EGFP (the EV) or DN CRTC1. B. Primary RPT cells were cotransfected with pH β 1-1141Luc and either CREB WT or CREB R314A. In A and B, the cultures were incubated 4 hrs with 280 nM PGE₂ or untreated. The level of luciferase was compared with levels in the untreated Control (EGFP in A and CREB WT in B). Values are averages of quadruplicate determinations $\pm$ SEM. * $p < 0.05$ relative to the untreated EGFP control (A) or the untreated CREB WT control (B).

Figure 4.

Regulation of Norepinephrine Stimulation by CRTCl. A. Primary RPT cells were cotransfected with pH β 1-1141Luc and either EGFP (the EV) or DN CRTCl. The cultures were incubated 4 hrs with 1 μ M norepinephrine or untreated. B. Primary RPT cells were cotransfected with pH β 1-1141Luc and either CREB WT or CREB R314A. The cultures were incubated 4 hrs with 1 μ M norepinephrine or untreated. C. Primary RPT cells were transfected with pH β 1-1141Luc. The cultures were incubated 4 hrs 1) untreated or with 1 μ M norepinephrine, 2) with 1 μ M prazosin +/- 1 μ M norepinephrine, 3) with 5 μ M yohimbine +/- 1 μ M norepinephrine, or 4) with 1 μ M propranolol +/- 1 μ M norepinephrine. D. Primary RPT cells with transfected with pH β 1-1141Luc. The cultures were incubated 4 hrs, with either 1) no supplement (i.e. untreated), 2) 1 μ M phenylephrine, 3) 1 μ M guanabenz, 4) 1 μ M albuterol, 5) 1 μ M metoprolol, or 6) 1 μ M norepinephrine. Luciferase activity was compared with levels in the untreated Control (i.e. EGFP in A, Control WT CREB in B, or untreated Control in C and D). Values are averages of quadruplicate determinations +/- SEM. * $p < 0.05$ as compared with the untreated EGFP control (A),

untreated WT-CREB control (B), or untreated Control (C and D). # $p < 0.05$ as compared with the untreated CREB R314A control (B).

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Figure 5.

Effect of Agonists on cAMP Production. Primary RPT cells in 48 well plates were incubated at 37°C in the presence of 0.1 mM IBMX and either 280 nM PGE₁, 280 nM PGE₂, 10⁻⁵ M norepinephrine, or no further supplement. After 5 min, intracellular cAMP was determined, and after 4 hrs, the cAMP in the culture medium was determined using an ELISA, as described in Materials and Methods. Values are averages $\pm$ SEM of determinations made in each of three parallel cultures.

Figure 6.

Effect of Agonists on Intracellular Ca^{2+} . Primary RPT cells in 96 well plates were loaded with Fluo-4-AM as described in Materials and Methods, and then incubated at 23°C with either A. 280 nM PGE_1 , B. 280 nM PGE_2 , C. 10^{-5} M norepinephrine, or D. 5 μM monensin. After excitation at 488 nm, the fluorescence emission at 530 nm was measured over 20 min, as described in Materials and Methods.

Figure 7.

Effect of H89 and KN62 on the Response to PGE₂ and Norepinephrine. Primary RPT cells were transiently transfected with pH β 1-1141Luc. A. The transfected cultures were incubated 4 hrs in the presence or absence of 10 μ M H89, as well with a) 280 nM PGE₂, b) 1 μ M norepinephrine, or c) no additive. B. The transfected cultures were incubated 4 hrs in the presence or absence of 10 μ M KN-62, as well as with a) 280 nM PGE₂, b) 1 μ M norepinephrine, or c) no additive. The level of luciferase was compared with levels in the untreated Control (-H89 in A and - KN62 in B). Values are averages of quadruplicate determinations $\pm$ SEM. * $p < 0.05$ relative to the untreated Control (untreated -H89) (A) or the untreated Control (untreated -KN62)(B).

Figure 8.

Regulation of Transcription by Dopamine. A. Primary RPT cells were transiently transfected with pH β 1-1141Luc. Subsequently, the cultures were incubated 4 hr with either 10 μ M dopamine (+/- 5 μ M monensin), 10 μ M L-dopa (+/- 5 μ M monensin), or in the presence or absence (+/-) of 5 μ M monensin. B. Primary RPT cells were transiently transfected with either the PGRE3 vector, or the PGRE3 GC Trans mutant. Subsequently, the cultures were treated with either dopamine or monensin, as described in Fig. 8A. C. Primary RPT cells were cotransfected either with pH β 1-1141Luc and the EV, or with the DN CRTC1. Subsequently, the cultures were incubated with either dopamine or monensin, as described in Fig. 8A. D. Primary RPT cells were transiently transfected with pH β 1-1141Luc. Subsequently, the cultures were incubated 4 hr in the presence, or absence of 10 μ M dopamine, and either 100 nM L741,626, 50 nM SCH23390, or with no additional compound. E. Primary RPT cells were transiently transfected with pH β 1-1141Luc. Subsequently, the cultures were incubated 4 hr with either 10 μ M dopamine, 10 μ M SKF 38,393, 2 μ M quinpirole, or untreated. Values are averages (+/- SEM) of luciferase activity, relative to the untreated Control. * p<0.05 relative to the untreated Control (A, B, C, D); # P<0.05 relative to the control in the same condition (i.e. either + dopamine or + L-Dopa, but without monensin); ¶p<0.05 relative to cultures treated with L741,626 alone.

Figure 9.

Role of SIK1 in Transcriptional Regulation. Primary RPT cells were transiently transfected with pH β 1-1141Luc and either the SIK KM expression vector, or the pSVL empty vector (EV). A. The transfected cultures were incubated 4 hrs either with 1 μ M norepinephrine, 280 nM PGE₁ or untreated. B. The transfected cultures were incubated 4 hrs either with 5 μ M monensin, 10 μ M dopamine, 5 μ M monensin and 10 μ M dopamine, or no further additive. Values are averages of quadruplicate determinations ($\pm$ SEM), relative to untreated Empty Vector (EV) Control. * $p < 0.05$ relative to untreated EV control; # $p < 0.05$ relative to untreated SIK KM Mutant; ; $\ddagger p < 0.05$ relative to monensin treated EV cultures.