Nuclear localization of regucalcin is enhanced in culture with protein kinase C activation in cloned normal rat kidney proximal tubular epithelial NRK52E cells

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Abstract. In this study we investigated whether the nuclear localization of regucalcin in cloned normal rat kidney tubular epithelial NRK52E cells is regulated after culture with hormonal signaling factors. Stable regucalcin/pCXN2 transfectants with subconfluent monolayers were further cultured for 24 or 48 h in a serum-free medium containing either vehicle, tumor necrosis factor-α (TNF-α), transforming growth factor-ß1 (TGF-ß1), parathyroid hormone (PTH), phorbol 12-myristate 13-acetate (PMA), or other factors. Culture with TNF-α (1.0 ng/ml of medium) or TGF-ß1 (5.0 ng/ml) for 48 h caused a significant decrease in regucalcin mRNA levels in NRK52E cells (wild-type), while regucalcin mRNA levels were markedly increased in the presence of PMA (10^{-6} M), an activator of protein kinase C, in wild-type cells. Immunocytochemical observation showed that HA-regucalcin was markedly localized in the nucleus of HA-regucalcin/phCMV2-transfected cells. The nuclear localization was enhanced in culture with BS (5%), PTH (10^{-7} M), Bay K 8644 ($2.5x10^{-6}$ M), or PMA (10^{-6} M) for 24 or 48 h. Culture with staurosporine, an inhibitor of protein kinase C, caused a remarkable decrease in the localization of HA-regucalcin in the nucleus of HA-RGPR-p117/phCMV2-transfected cells with PMA. Culture with PMA (10^{-6} M) for 24 or 48 h caused a remarkable increase in nuclear regucalcin protein levels. The effect of PMA in increasing nuclear regucalcin levels was completely absent in culture with staurosporine (10^{-8} M). The nuclear localization of regucalcin in the stable regucalcin/pCXN2-transfected cells (transfectant) increased markedly as compared with that of wild-type cells, whereas the increase was less evident in the transfectants cultured with staurosporine. This study demonstrated that regucalcin localizes in the nucleus of cloned normal rat kidney proximal tubular epithelial NRK52E cells, and that its nuclear localization is enhanced through an intracellular signaling process which involves protein kinase C.

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Introduction

Regucalcin was discovered as a novel Ca^{2+}-binding protein not including the EF-hand motif, in the liver cytosol of rats (1-3). The name regucalcin was proposed for this Ca^{2+}-binding protein, which can regulate the effects of Ca^{2+} and/or calmodulin on various enzymes in liver cells (4,5).

Past studies demonstrated that regucalcin plays a multifunctional role as a regulatory protein in the intracellular signaling pathway in many cell types (6-8). Regucalcin has been shown to play a role in the maintenance of intracellular Ca^{2+} homeostasis and in the inhibitory regulation of various Ca^{2+}-dependent protein kinases, tyrosine kinases, and protein phosphatases, in nitric oxide synthase, and in the suppression of nuclear DNA and RNA syntheses (6-8). Regucalcin has also been demonstrated to have a suppressive effect on cell proliferation and apoptotic cell death that is mediated through various extracellular signaling factors (9-11). It has been proposed that regucalcin plays a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation (8).

The expression of regucalcin mRNA and its protein has been demonstrated in cloned normal rat kidney proximal tubular epithelial NRK52E cells *in vitro*, and mRNA expression is enhanced after the stimulation of hormones that are related to ion transport in the kidney proximal tubular epithelial cells *in vitro* (12). The overexpression of regucalcin has been shown to cause a remarkable increase in its localization in the nucleus of NRK52E cells (13), and it also has been shown to suppress the expression of L-type Ca^{2+} channel and calcium-sensing receptor mRNA in NRK52E cells (13). However, whether regucalcin has direct or indirect effects on their gene expression in the nucleus is uncertain. We speculated that the nuclear localization of regucalcin in NRK52E cells plays an important role in the regulation of gene expression.

This study, therefore, was undertaken to determine whether the nuclear localization of regucalcin is enhanced after culture with hormone action and its intracellular signaling factors, using cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin. We found that the nuclear localization of regucalcin is enhanced in NRK52E cells cultured with PMA, an activator of protein kinase C.

Materials and methods

Chemicals. Non-essential amino acid solution, bovine serum (BS), and penicillin-streptomycin solution (5,000 U/ml penicillin, 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Dulbecco's minimum essential medium (DMEM), anti-HA-tag antibody, Bay K 8644, PTH [synthetic human parathyroid hormone (1-34)], tumor necrosis factor-α, transforming growth factor-ß1 (TGF-ß1), staurosporine, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemicals Co. (St. Louis, MO). The following materials were used: restriction enzymes (Takara Shuzo Co., Ohtsu, Japan), phCMV2 vector (Gene Therapy Systems, Inc., San Diego, CA), and fluorescein-conjugated affinity purified secondary antibody (Chemicon International, Temecula, CA). Other chemicals were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Reagents were dissolved in distilled water or ethanol, and some were passed through an ion-exchange resin to remove metal ions.

Cell culture. The cloned normal rat kidney proximal tubular epithelial cells (NRK52E; $1x10^5$) (14) were maintained in DMEM supplemented with 5% BS, non-essential amino acid solution, 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37˚C to obtain subconfluent monolayers.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. NRK52E cells (wild-type) with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), TGF-ß1 (1.0 or 5.0 ng/ml), or PMA (10^{-6} M) without BS. After culture, total RNAs in the cells were prepared using Trizol. RT-PCR was performed with a Titan™ One-Tube RT-PCR kit (Roche Molecular Biochemicals), as recommended by the supplier. Primers used for the amplification of regucalcin cDNA were: 5'-GGAGGC TATGTTGCCACCATTGGA-3' (sense strand, position 292-314 of cDNA sequence) and 5'-CCCTCCAAAGCAGCATG AAGTTG-3' (antisense strand, position 849-827 of cDNA sequence) (3). For semiquantative PCR, G3PDH was used as an internal control to evaluate total RNA input. Primers used for the amplification of G3PDH were: 5'-GATTTGGCCGTA TCGGACGC-3' (sense strand) and 5'-CTCCTTGGAGGCC ATGTAGG-3' (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977-bp sequence from the mRNA of G3PDH.

RT-PCR was performed using a reaction mixture (20 μl) containing 2 μg of total RNAs, supplied RT-PCR buffer, Titan enzyme mix (AMV and Expand High Fidelity; 0.2 mM dNTP, 5 mM DTT, 5U RNase inhibitor), and 0.3 μM primers. Samples were incubated at 50˚C for 30 min, then amplified for 35 cycles under the following conditions: denaturation for 30 sec at 94˚C, annealing for 30 sec at 60˚C or 62˚C, and extension for 60 sec at 68˚C. The amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining.

Plasmid constructs. For the construction of the HA-tagged regucalcin expression vector, DNA covering the coding region of regucalcin was amplified by PCR using rat liver cDNA as a template (3,15). The cDNA corresponding to the nucleotides was amplified by PCR using a sense primer flanked by *Bgl*III and antisense primer flanked by *Hin*dIII. The primer sequences were: sense primer, 5'-AAGATCT CATGTCTTCCATCAAGATTGA-3', and antisense primer, 5'-CCCTCCAAAGCAGCATGAAGTTG-3'. The amplified DNA fragments were digested with *Bgl*II and *Hin*dIII. The amplified regucalcin/pCXN2 was digested with *Hin*dIII and *Not*I. These digested fragments were inserted into the *Bgl*II-*Hin*dIII sites of the phCMV2 vector containing HA to generate HA-regucalcin/phCMV2 vector. All PCRs were performed with a Takara PCR Thermal Cycler MP (Takara Shuzo Co.) for 30 cycles under the following conditions: 94˚C for 1 min, 55-58˚C for 1 min, and 72˚C for 1 min. All constructs were sequenced to ensure the accuracy of reading frames using a fluorescence DNA sequencer (ABI Prism 310-2 Genetic Analyzer; PerkinElmer Applied Biosystems, Foster City, CA)

Confocal microscopy imaging. For a transient transfection assay, NRK52E cells were grown on 2-well chamber slides to approximately 70% confluence. The cells were then transfected with the expression vector, HA-regucalcin/phCMV2, using synthetic cationic lipid components, a lipofectamine (Invitrogen), according to the manufacturer's instructions (15), and cultured for 24-48 h in DMEM containing either vehicle, BS (5%), PTH (10^{-7} M), Bay K 8644 ($2.5x10^{-6}$ M), PMA (10^{-7} M), staurosporine (10^{-8} M), or PMA (10^{-7} M) plus staurosporine (10^{-8} M) in the absence of BS.

The transfected cells were fixed with 4% paraformaldehyde in PBS for 30 min, washed once with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 30 min. After washing with PBS, they were blocked with Block Ace (Dainippon Pharmaceutical Co., Suita, Japan) for 1 h, then incubated with anti-HA-tag antibody (Sigma) diluted 1:100 in Block Ace solution, at 4˚C overnight. After incubation with the primary antibody, the cells were washed 3 times with PBS, then incubated for 2 h with fluorescein-conjugated anti-mouse IgG diluted (1:100) in Block Ace solution at room temperature (16). Subsequently, the cells were washed with PBS, coverslipped by Vectashield, and observed with an MRC-1024 Confocal Laser Scanning Imaging System (Bio-Rad).

Western blot analysis. NRK52E cells (wild-type) or stable regucalcin/pCXN2 transfectant cells (12) were cultured for 72 h in DMEM containing 5% BS and after a change of medium, the cells were cultured for 24 or 48 h in a medium containing either vehicle, PTH (10^{-7} M), PMA (10^{-6} M), staurosporine (10^{-8} M), or PMA (10^{-6} M) plus staurosporine (10^{-8} M). After culture, the cells were washed 3 times with

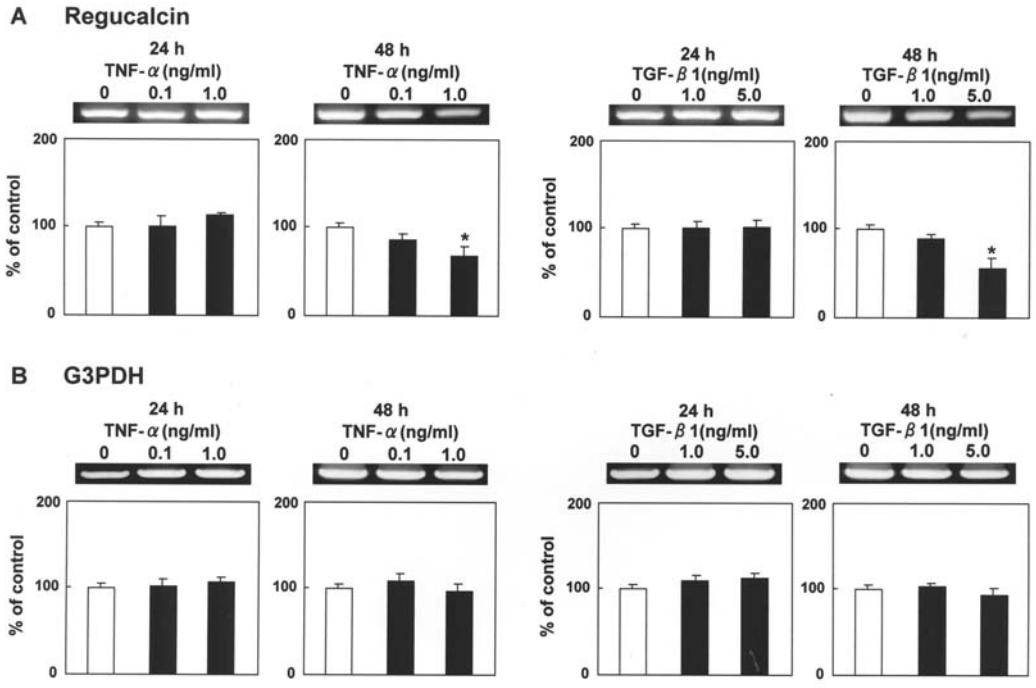

Figure 1. Effects of TNF-α or TGF-ß1 on regucalcin mRNA expression in cloned normal rat kidney proximal tubular epithelial NRK52E cells. NRK52E cells (wild-type) were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to a medium containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-ß1 (1.0 or 5.0 ng/ml) without BS. After the medium change, the cells were cultured for 24 or 48 h. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for regucalcin (A) or G3PDH (B) cDNAs. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA level were indicated as a percentage of control (the mean ± SEM of three or four experiments with separate cell samples). *p<0.01 compared with the value obtained from wild-type cells.

phosphate-buffered saline (PBS), scraped into 0.2 ml of ice-cold 0.25 M sucrose solution and disrupted for 30 sec with an ultrasonic device. The scraped cells were centrifuged at 500 x g for 5 min to remove cell debris. The 500 x g supernatant was centrifuged at 1000 x g for 10 min to precipitate the nuclear fraction. The 1000 x g supernatant was used as the supernatant fraction, and the 1000 x g precipitate was suspended in 0.25 M sucrose and pooled to analyze regucalcin by Western blot analysis. The protein concentration was determined by the method of Lowry *et al* (17). Aliquots of protein (18 μg) were mixed with 5X Laemmli sample buffer and boiled for 5 min, and SDS-PAGE was performed by the Laemmli method using 7.5% polyacrylamide resolving gel (18). After SDS-PAGE, the proteins were then transferred onto polyvinylidene difluoride membranes at 100 mA for 4 h. The membranes were incubated with anti-HA-tag antibody, which was diluted 1:1000 in 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 0.1% (w/v) Tween-20 (washing-buffer), and 5% skimmed milk for 1 h. The membranes were incubated for 1 h with horseradish peroxidase-linked anti-mouse IgG, which was diluted 1:2000 with washing buffer containing 5% skimmed milk, then washed again. The detection of protein bands was performed using an enhanced chemiluminescence kit according to the manufacturer's instructions. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

Statistical analysis. Data are expressed as the mean ± SEM. Statistical differences were analyzed using a Student's t-test, and p-values <0.05 were considered to indicate statistical significance. We also used an ANOVA multiple comparison test to compare the treatment groups.

Results

Effect of TNF-α, TNF-β1, or PMA on regucalcin mRNA expression in cloned NRK52E cells. Cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) were cultured for 72 h in a medium containing 5% BS. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, TNF-α (0.1 or 1.0 ng/ml of medium), or TGF-ß1 (1.0 or 5.0 ng/ml) in the absence of BS. Cultures with TNF-α (1.0 ng/ml) or TGF-ß1 (5.0 ng/ml) for 48 h caused a significant decrease in regucalcin mRNA levels (Fig. 1A). G3PDH mRNA levels were not significantly changed in the presence of TNF-α (0.1 or 1.0 ng/ml) or TGF-ß1 (1.0 or 5.0 ng/ml).

Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle or PMA (10^{-6} M) without BS (Fig. 2). Regucalcin mRNA levels increased significantly in the presence of PMA (10^{-6} M), an activator of protein kinase C (Fig. 2A). G3PDH mRNA levels did not change significantly in the presence of PMA (10^{-6} M) (Fig. 2B).

Nuclear localization of regucalcin in cloned NRK52 cells. Cloned NRK52E cells were cultured for 72 h in a medium containing 5% BS. Cells with subconfluency were transiently cultured for 24 or 48 h in a medium containing either vehicle, BS (5%), PTH (10^{-7} M), Bay K8644 ($2.5x10^{-6}$ M), PMA (10^{-6} M), staurosporine (10^{-8} M), or PMA (10^{-6} M) plus staurosporine (10^{-8} M) without BS (Fig. 3). Immunocyto-

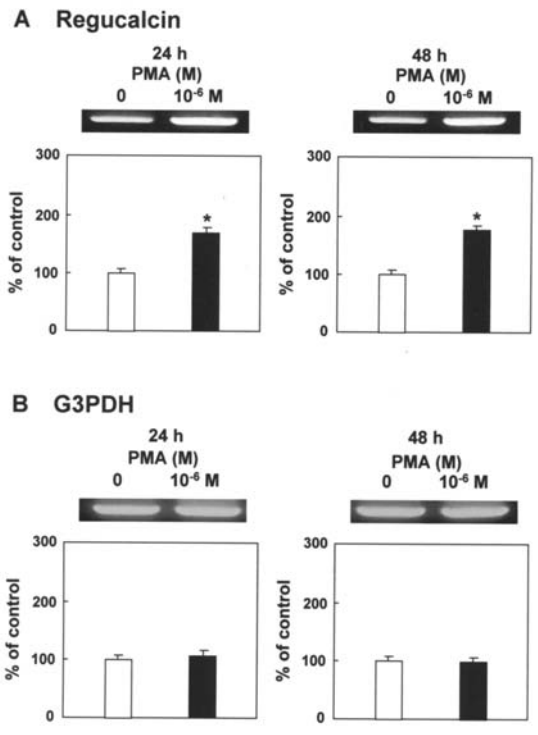

Figure 2. Effects of PMA on regucalcin mRNA expression in cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type). NRK52E cells (wild-type) were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to a culture medium containing either vehicle or PMA (10^{-6} M) without BS. After the medium change, the cells were cultured for 24 or 48 h. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for regucalcin (A) or G3PDH (B) cDNAs. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA level were indicated as a percentage of control (the mean ± SEM of three or four experiments with separate cell samples). $^{*}p<0.01$ compared with the value obtained from wild-type cells.

chemical observation showed that HA-regucalcin was localized in the nucleus of HA-regucalcin/phCMV2-transfected cells (Fig. 3A). The nuclear localization was enhanced after culture with BS, PTH, Bay K 8644, or PMA for 24 or 48 h (Fig. 3B and C). The presence of staurosporine, an inhibitor of protein kinase C, caused a notable decrease in the localization of HA-regucalcin in the nucleus of HA-RGPR-P117/phCMV2-transfected cells (Fig. 3D). Whether a significant enhancement of nuclear regucalcin localization caused the change in cell structure is unknown.

Wild-type NRK52E cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, PTH (10^{-7} M), or PMA (10^{-6} M) without BS (Fig. 4A). Regucalcin protein levels in the nuclear fraction were determined using Western blot analysis. Culture with PMA (10^{-6} M) for 24 or 48 h caused a notable increase in nuclear regucalcin levels. Culture with PTH (10^{-7} M) for 48 h caused a significant increase in nuclear regucalcin levels. PMA therefore had a potent effect on nuclear regucalcin levels in the NRK52E cells.

When the wild-type cells with subconfluency were cultured for 24 h in a medium containing either vehicle, PMA (10^{-6} M), staurosporine (10^{-8} M), or PMA (10^{-6} M) plus staurosporine (10^{-8} M) (Fig. 4B), the effect of PMA in increasing nuclear regucalcin levels was completely absent.

The nuclear localization of regucalcin in the stable regucalcin/pCXN2-transfected cells (transfectant) was examined (Fig. 4C). Wild-type cells or transfectants were cultured for 24 h in a medium containing either vehicle or staurosporine (10^{-8} M) without BS. In the absence of staurosporine, regucalcin levels in the supernatants (including mitochondria, microsomes, and cytoplasm) or the nuclear fraction increased markedly in the transfectants as compared

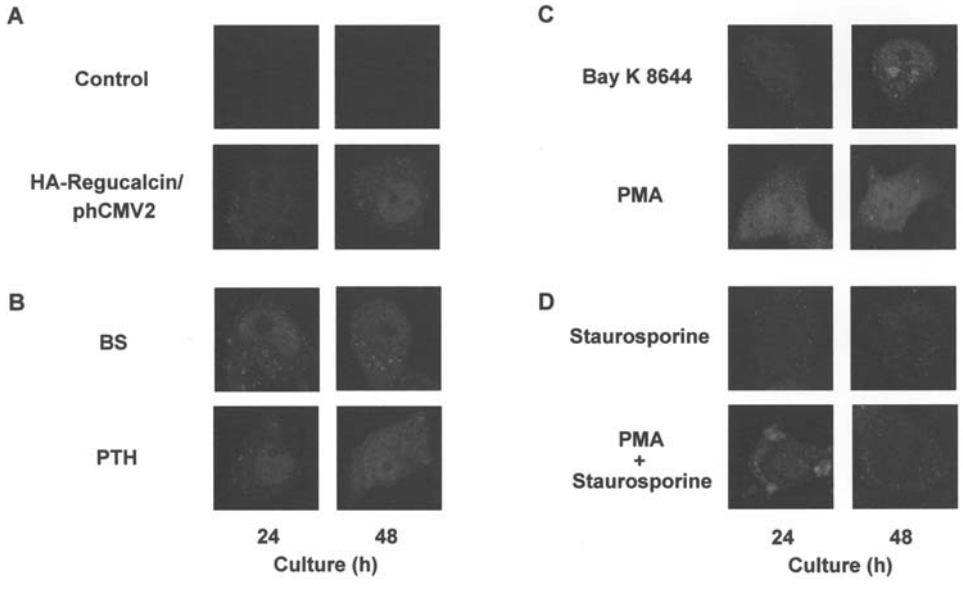

Figure 3. Analysis of the subcellular localization of HA-regucalcin in cloned normal rat kidney proximal tubular epithelial NRK52E cells. NRK52E cells (wild-type) were cultured for 72 h in a medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to a culture medium without BS. NRK52E cells with subconfluency were transiently transfected with HA-regucalcin/phCMV2, and wild-type NRK52E cells or transfectants were cultured in DMEM without BS for 24 or 48 h (A). NRK52E cells transiently transfected with HA-regucalcin/phCMV2 were cultured in DMEM with either BS (5%), PTH (10^{-7} M), Bay K 8644 ($2.5x10^{6}$ M), PMA (10^{-6} M), staurosporine (10^{-8} M), or PMA (10^{-6} M) plus staurosporine (10^{-8} M) for 24 or 48 h (B-D). After culture, the cells were processed for immunocytochemical observation using confocal fluorescence microscopy. The figure shows one of 3-6 experiments with separate samples.

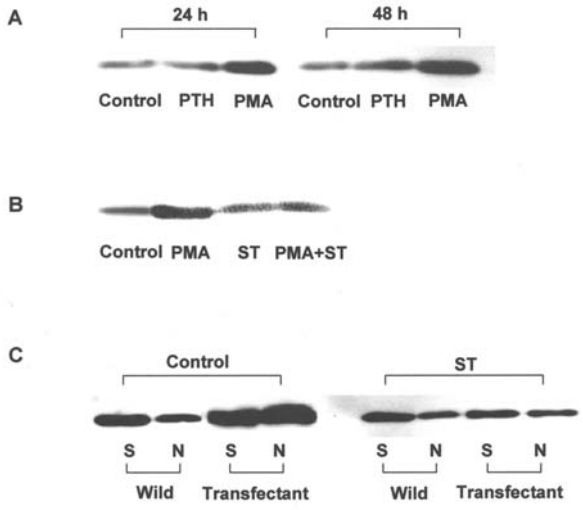

Figure 4. Western blot analysis of regucalcin localization in the nucleus of NRK52E (wild-type) or stable regucalcin/pCXN2-transfected cells (transfectant). (A) After culture for 72 h with 5% BS, NRK52E cells (wild-type) with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, PTH (10^{-7} M), or PMA (10^{-6} M) in the absence of BS. (B) After culture for 72 h with 5% BS, cells (wild-type) with subconfluency were cultured for 24 h in a medium containing either vehicle, PMA (10^{-6} M), staurosporine (ST; 10^{-8} M), or PMA (10^{-6} M) plus staurosporine (10^{-8} M) in the absence of BSA. (C) After culture for 72 h with 5% BS, wild-type cells or stable regucalcin/pCXN2-transfected cells (transfectant) with subconfluency were cultured for 24 h in a medium containing either vehicle or staurosporine (10^{-8} M) in the absence of BS. The nucleus (N) and the 1,000 x g supernatant (S) (containing mitochondria microsomes, and cytoplasm) were prepared from the cell lysate. Regucalcin was analyzed using Western blotting with an anti-regucalcin antibody.

with the wild-type cells. These increases were less evident in the transfectants cultured with staurosporine.

Discussion

The expression of regucalcin mRNA in cloned normal rat kidney proximal tubular epithelial NRK52E cells has been shown to increase after culture with PTH or aldosterone (12). Furthermore, we examined the effect of TNF-α, TNF-ß1, or PMA, on regucalcin mRNA expression in cloned NRK52E cells. We found that regucalcin mRNA expression in NRK52E cells was markedly enhanced after culture with PMA, an activator of protein kinase C (19), while its expression was suppressed after culture with TNF-α or TGF-ß1. PMA has been demonstrated to have a stimulatory effect on regucalcin mRNA expression in cloned rat hepatoma H4-II-E cells (20). The present finding supports the view that regucalcin mRNA expression is mediated through Ca^{2+} signaling factors with the involvement of protein kinase C.

The suppression of regucalcin mRNA expression has not been fully clarified. Culture with TNF-α or TGF-ß1 for 48 h was found to induce a significant decrease in regucalcin mRNA levels in NRK52E cells (wild-type), suggesting that TNF-α- or TGF-ß1-related signaling factors are involved in the suppression of reguclacin mRNA expression. The finding that regucalcin mRNA expression is suppressed after culture with TNF-α or TGF-ß1 is novel, however, its significance remains to be elucidated.

Immunocytochemical observation showed that HA-regucalcin was localized in the nucleus of HA-regucalcin/phCMV2-transfected cells. The nuclear localization was enhanced in cultures with BS, PTH, Bay K 8644, or PMA. This enhancement was marked in culture with PMA. PMA-enhanced regucalcin expression was significantly suppressed in HA-regucalcin/phCMV2-transfected cells cultured with staurosporine, an inhibitor of protein kinase C. These observations suggest that regucalcin, which is enhanced through Ca^{2+}-signaling factors including protein kinase C, is localized in the nucleus of cloned normal rat kidney proximal tubular epithelial NRK52E cells.

The results of Western blot analysis indicate that nuclear regucalcin levels increased markedly in culture with PMA. This increase was diminished in the presence of staurosporine, an inhibitor of protein kinase C. Thus, the results of Western blot analysis supported the immunocytochemical observation of the nuclear localization of regucalcin in cloned NRK52E cells.

The nuclear localization of regucalcin in the stable regucalcin/pCXN2-transfected cells (transfectants) was markedly higher than in wild-type cells. This increase was not seen in the transfectants cultured with staurosporine. These observations may support the view that the localization of regucalcin in the nuclei is enhanced through Ca^{2+} signaling related to protein kinase C in NRK52E cells.

The expression of regucalcin mRNA in NRK52E cells may be partly mediated through signaling systems that are related to cyclic AMP or protein kinase. It has been shown that the action of PTH is mediated through cyclic AMP or inositol 1,4,5-trisphosphate (IP3)-released Ca^{2+} and protein kinase C in cells (21). It is assumed that the effect of PTH in stimulating the nuclear localization of regucalcin in NRK52E cells may be mediated through cyclic AMP and/or Ca^{2+}-dependent protein kinase C in NRK52E cells. In addition, PMA, an activator of protein kinase C, was found to markedly enhance the nuclear localization of regucalcin in NRK52E cells. Protein kinase C may play a pivotal role in the enhancement of regucalcin mRNA expression and the nuclear localization of regucalcin protein in NRK52E cells.

The nuclear localization of regucalcin in NRK52E cells was enhanced through intracellular signaling factors. It is possible that hormonal signaling is partly mediated through the expression of regucalcin. The overexpression of regucalcin has been shown to regulate the expression of various genes in NRK52E cells (11,13). We surmise that the nuclear localization of regucalcin, which is enhanced through hormonal signaling, has a role in the regulation of gene expression in the nucleus.

In conclusion, we demonstrated that regucalcin localizes in the nucleus of the cloned normal rat kidney proximal tubular epithelial NRK52E cells, and that its nuclear localization is enhanced through the intracellular signaling of protein kinase C. Regucalcin may play a regulatory role in gene expression in the nucleus of NRK52E cells.

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