Journal of Cellular Biochemistry 99:1064–1077 (2006)

Overexpression of Regucalcin Enhances Its Nuclear Localization and Suppresses L-type Ca^{2+} Channel and Calcium-Sensing Receptor mRNA Expressions in Cloned Normal Rat Kidney Proximal Tubular Epithelial NRK52E Cells

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Abstract The effect of regucalcin (RC), a regulatory protein in intracellular signaling pathway, on the gene expression of various mineral ion transport-related proteins was investigated using the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing RC. NRK52E cells (wild-type) and stable RC/pCXN2 transfectant were cultured for 72 h in medium containing 5% bovine serum (BS) to obtain subconfluent monolayers. After culture for 72 h, cells were further cultured 24–72 h in a medium containing either vehicle, aldosterone (10^{-8} or 10^{-7} M), or parathyroid hormone (PTH) (1–34) (10^{-8} or 10^{-7} M) without BS. RC was markedly localized in the nucleus of transfectants. Overexpression of RC caused a significant increase in rat outer medullary K^+ channel (ROMK) mRNA expression, while it caused a remarkable decrease in L-type Ca^{2+} channel and calcium-sensing receptor (CaR) mRNA expressions. Overexpression of RC did not have an effect on epithelial sodium channel (ENaC), Na, K-ATPase (alpha-subunit), Type II Na-Pi cotransporter (NaPi-IIa), angiotensinogen, Na^+-Ca^{2+} exchanger, and glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) mRNA expressions. Hormonal effect on gene expression, moreover, was examined. Culture with aldosterone (10^{-8} or 10^{-7} M) caused a significant increase in ENaC, Na, K-ATPase, and ROMK mRNA expressions in the wild-type cells. Those increases were weakened in the transfectants. Culture with PTH (10^{-8} or 10^{-7} M) significantly decreased NaPi-IIa mRNA expression in the wild-type cells. This effect was not altered in the transfectants. PTH significantly decreased angiotensinogen mRNA expression in the wild-type cells and the transfectants, while aldosterone had no effect. Culture with PTH (10^{-8} or 10^{-7} M) caused a significant decrease in L-type Ca^{2+} channel and CaR mRNA expressions in the wild-type cells, while the hormone significantly increased Na^+-Ca^{2+} exchanger mRNA expression. The effects of PTH on L-type Ca^{2+} channel, CaR, and Na^+-Ca^{2+} exchanger mRNA expressions were also seen in the transfectants. This study demonstrates that overexpression of RC caused a remarkable increase in its nuclear localization, and that it has suppressive effects on the gene expression of L-type Ca^{2+} channel or CaR, which regulates intracellular Ca^{2+} signaling, among various regulator proteins for mineral ions in NRK52E cells. J. Cell. Biochem. 99: 1064–1077, 2006.

Key words: regucalcin; nuclear localization; L-type Ca^{2+} channel; calcium-sensing receptor; transcription; kidney NRK52E cells

Regucalcin (RC) was discovered as a novel Ca^{2+}-binding protein not including the EF-hand motif, which differs from calmodulin, in the liver cytosol of rats [Yamaguchi and Yamamoto, 1978]. The name RC was proposed for this Ca^{2+}-binding protein, which can regulate the Ca^{2+} or/and calmodulin effects on various enzymes in liver cells [Yamaguchi and Mori, 1988; Yamaguchi, 1992; review]. In recent years, RC has been demonstrated to play multifunctional role as a regulatory protein in intracellular signaling pathway in many cell types [Yamaguchi, 2000a,b, 2005; review].

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Received 15 November 2005; Accepted 11 January 2006
DOI 10.1002/jcb.20863

We have cloned cDNA for RC from human, rat, mouse, rabbit, bovine, chicken, and toad livers [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The nucleotide and amino acid sequences of RC from seven vertebrate species were highly conserved in their coding region, and they had 69.9–91.3 identity [Misawa and Yamaguchi, 2000]. The rat and human RC genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. RC are greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of RC mRNA is mediated through Ca^{2+}-sensing mechanism that is stimulated with various hormones [Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999]. AP-1, NFI-A1, and RGPR-p117 (a novel protein which is related to the RC gene promoter region) have been found to be transcriptional factors for the enhancement of RC gene promoter activity [Murata and Yamaguchi, 1998, 1999; Misawa and Yamaguchi, 2001, 2002; Sawada and Yamaguchi, 2005].

In recent years, RC has been proposed to play a physiologic role in maintaining homeostasis and functions as a regulatory protein on intracellular signaling process in many cell types [Yamaguchi, 2000a,b, 2005; reviews]. RC plays an important role in the maintenance of intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinases and thyrosine kinases, protein phosphatases, nitric oxide (NO) synthase, protein synthesis, and DNA and RNA syntheses enhanced in proliferative cells [Tsurusaki and Yamaguchi, 2002a,b; Izumi et al., 2003]. RC has been shown to translocate to the nucleus of rat liver, and it has been demonstrated to regulate nuclear function in regenerating rat liver with proliferative cells [Tsurusaki et al., 2000]. Overexpression of RC has a suppressive effect on DNA synthesis and cell proliferation in the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002; Yamaguchi and Daimon, 2005]. Moreover, overexpression of RC has been shown to have a suppressive effect on cell death and apoptosis in the cloned rat hepatoma H4-II-E cells [Izumi and Yamaguchi, 2004a,b]. Thus, RC has been proposed to play a physiologic role in maintaining homeostasis of cellular response for cell stimulation [Yamaguchi, 2005; review]. Interestingly, RC transgenic rats have been shown to induce bone loss and hyperglycemia [Yamaguchi et al., 2002, 2004], suggesting its pathophysiologic role.

RC is greatly expressed in liver, and its relatively higher levels are also found in kidney cortex [Yamaguchi and Isogai, 1993; Yamaguchi and Kurota, 1995]. RC may play a physiologic role in the regulation of cell function in kidney cortex cells, which participate in reabsorption of calcium and other ions in kidney proximal tubular epithelial cells [Van Os, 1987]. RC has been shown to play a role in the regulation of intracellular Ca^{2+} transport [Kurota and Yamaguchi, 1997a,b; Xue et al., 2000], and this protein has an inhibitory effect on Ca^{2+}-dependent protein kinases [Kurota and Yamaguchi, 1997c], protein phosphatases [Morooka and Yamaguchi, 2002], and NO synthase [Ma and Yamaguchi, 2003] in the cytosol and nucleus of rat kidney cortex. Moreover, RC has been shown to express in the cloned normal rat kidney proximal tubular epithelial NRK52E cells, and its mRNA expression has been demonstrated to stimulate by parathyroid hormone (PTH) or aldosterone, which targets kidney proximal tubular epithelial cells [Nakagawa and Yamaguchi, 2005a]. Overexpression of RC has been shown to suppress cell proliferation [Nakagawa et al., 2005] and apoptotic cell death [Nakagawa and Yamaguchi, 2005b] in kidney NRK52E cells. Thus, RC plays an important role in the regulation of kidney cell function.

This study was undertaken to determine whether RC localizes in the nucleus and regulates gene expression of various proteins that involve in mineral ion transport in the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing RC.

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 modified Eagle's medium (DMEM), parathyroid hormone (PTH), synthetic human PTH (1–34) and D-aldosterone were purchased from Sigma (St. Louis, MO). Other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan). Reagents used were dissolved in distilled water or ethanol.

Cell Culture

The cloned normal rat kidney proximal tubular epithelial cells (NRK52E; 1×10^5 cells) [Rice et al., 2003] were maintained for 72 h in DMEM supplemented 5% BS, non-essential amino acid, and 50 U/ml penicillin and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain subconfluent monolayers. After culture, cells were washed three times with phosphate-buffered saline (PBS), and the cells were incubated for 24, 48, or 72 h in DMEM without 5% BS in the absence or presence of aldosterone (10^{-8} or 10^{-7} M) or PTH (10^{-8} or 10^{-7} M). Cells were washed three times with PBS after culture.

Regucalcin Transfectants

The cDNA encoding rat RC was isolated and cloned into the pBluescript vector [Shimokawa and Yamaguchi, 1993]. The RC cDNA contained *P*st I site downstream of the translational stop codon, and *P*st I site and an *Eco* RI upstream of the RC cDNA. The *Eco* RI fragment (containing the complete coding cDNA) was cloned into the *Eco* RI site of the pCXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as RC/pCXN2 [Misawa et al., 2002].

For transient transfection assay, the NRK52E cells were grown on 35-mm dishes to approximately 70% confluence. Each of RC/pCXN2 and pCXN2 vector alone was transfected into NRK52E cells using the synthetic cationic lipid components, a Tfx-20 reagent, according to the manufacturer's instructions (Promega) [Misawa et al., 2002]. At 48 h after transfection, cells were harvested and used for subsequent experiments. NRK52E cells were transfected with RC/pCXN2 vector alone using a Tfx-20 reagent. After 24 h, neomycin (1.0 mg/ml Geneticin G418, Sigma) was added to cultures for selection and cells were plated at limiting dilution. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in the medium without neomycin. RC was stably expressed in the transfectants. In experiments, transfectants were cultured for 24–72 h in DMEM containing 5% BS.

Cell Counting

After trypsinization of each of the culture dishes using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min at 37°C, cells were collected and centrifuged in a PBS at 100*g* for 5 min. The cells were resuspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a hemocytometer plate. For each dish, we took the average of two countings.

Preparation of Subcellular Fraction

The cells cultured were polled and scraped into 0.5 ml of ice-cold 0.25 M sucrose, and disrupted for 30 s with an ultrasonic device. Scrapped cells were also homogenized in Potter–Elvehjem homogenizer with a teflon pestle. The homogenates were spun at 100*g* in a refrigerated centrifuge for 5 min to obtain the nucleus. The 100 g supernatant fraction was spun at 1,000 g for 10 min, and the precipitated fraction (containing nucleus) and the resultanting supernatant (containing mitochondria, microsomes, and cytoplasm) were pooled. DNA concentration in the 1,000 g precipitated fraction was determined using the method of Ceriotti [1955]. Protein concentration in the nucleus and the supernatant fractions was measured using the method of Lowry et al. [1951].

Western Blot Analysis

The nucleus and the supernatant fractions were used for Western blot analysis [Wessendolf et al., 1993]. Aliquots of protein (10 μg) were mixed with 5× Laemmli sample buffer, boiled for 5 min, and SDS-PAGE was performed by the method of Laemmli [1970] using 12% polyacrylamide resolving gel. After SDS-PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 3 h. The membranes were incubated with a polyclonal rabbit anti-RC antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% (w/v) Tween-20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit IgG, which was diluted 1:5,000 with washing buffer containing 5% (w/v) skim milk and again they were washed. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacturer's instruction. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

Preparation of RNA

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. Kidney NRK52E cells cultured were homogenized in buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isopropanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

Determination of Specific mRNA by RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a Titan™ One Tube RT-PCR Kit (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the supplier to investigate the gene expression. The primers were based on the published rat sequences. Primers for epithelial sodium channel (ENaC) cDNA were: 5′-GCCACTCCAGAGAAGCTCAA-3′ (sense strand, positions 246–265 of cDNA sequence) and 5′-GCCCTGCTCTTCACGTTTGT-3′ (anti-sense strand, positions 504–523) [Canessa et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 278-bp sequence from the mRNA of rat ENaC. Primers for Na, K-ATPase (alpha-subunit) cDNA were: 5′-TATGTCTGACGCTCACTGCC-3′ (sense strand, positions 1,082–1,101 of cDNA sequence) and 5′-CTGCACGCTTAAGGATAGGC-3′ (anti-sense strand, positions 1,337–1,356) [Schneider et al., 1985]. The pair of oligonucleotide primers was designed to amplify a 274-bp sequence from the mRNA of rat Na, K-ATPase (alpha-subunit) cDNA. Primers for rat outer medullary K^+ channel (ROMK) cDNA were: 5′-GCCCACTTGGATCCTCACTA-3′ (sense strand, positions 1,319–1,338 of cDNA sequence) and 5′- CATGGACCCAGACTCAGACA-3′ (anti-sense strand, positions 1,509–1,528) [Ho et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 190-bp sequence from the mRNA of rat ROMK cDNA. Primers for rat Type II Na-Pi cotransporter (NaPi-IIa) cDNA were: 5′-GCCCTCACAGAGCACAGCT-3′ (sense strand, positions 249–297 of cDNA sequence) and 5′-GCTCTGCACCAGAACTGTCA-3′ (anti-sense strand, positions 510–529) [Magagnin et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 262-bp sequence from the mRNA of rat NaPi-IIa cDNA. Primers for rat angiotensinogen cDNA were: 5′-TTGGTTCACCAGGGGATAGC-3′ (sense strand, positions 125–144 of cDNA sequence) and 5′-TCTAGCTTCTCAGTGGCCAG-3′ (anti-sense strand, positions 481–500) [Bouhnik et al., 1981]. The pair of oligonucleotide primers was designed to amplify a 375-bp sequence from the mRNA of rat angiotensinogen cDNA. Primers for rat L-type Ca^{2+} channel cDNA were: 5′-CCAGCCCAGAAAAGAAACAG-3′ (sense strand, positions 3,099–3,018 of cDNA sequence) and 5′- CTGCCTTTTCCTTAAGGTGC-3′ (anti-sense strand, positions 3,349–3,368) [Gu et al., 1999]. The pair of oligonucleotide primers was designed to amplify a 270-bp sequence from the mRNA of rat L-type Ca^{2+} channel cDNA. Primers for calcium-sensing receptor (CaR) cDNA were: 5′-TCCATTTTGGAGTAGCAGCC-3′ (sense strand, positions 652–671) of cDNA sequence) and 5′-GCAATGGTCGAAGGGATGTG-3′ (anti-sense strand, positions 893–912) [Riccardi et al., 1995]. The pair of oligonucleotide primers was designed to amplify a 260-bp sequence from the mRNA of rat CaR cDNA. Primers for Na^+-Ca^{2+} exchanger cDNA were: 5′-AGGGACCAAGATGACGAGGA-3′ (sense strand, positions 934–953 of cDNA sequence) and 5′-GTGAGGGCCACAGTACCACA-3′ (anti-sense strand, positions 1,274–1,293) [Low et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 360-bp sequence from the mRNA of rat Na^+-Ca^{2+} exchanger cNDA. Primers for glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) cDNA were: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense strand) from the G3PDH Amplimer Set (Chonteck, Palo Alto, CA).

RT-PCR was performed using 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 deoxynucleotide triphosphate, 5 mM dithiothreitol, 5 U RNase inhibitor, 2.5 U Taq DNA polymerase, and 0.3 μM primers. Samples were incubated at 50°C for 30 min and at 94°C for 2 min, and then amplified for 35 cycles under the following conditions; denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s at 68°C. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining and quantitated using a densitometer.

Statistical Analysis

Data were expressed as the mean ± SEM. The significance of difference between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. A *P*-value of <0.05 was considered to indicate statistically significant difference.

RESULTS

Nuclear Localization of Regucalcin in Kidney NRK52E Cells

The cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or RC/pCXN2-transfected NRK52E cells (transfectants) were cultured for 72 h to reach subconfluent monolayers in the presence of 5% BS. Cells with subconfluency were cultured for 24 or 48 h in the absence of BS. The change in RC contents in the nuclear and the 1,000 g supernatant fractions (including mitochondria, microsomes, and cytoplasm) in wild-type cells and transfectants is shown in Figure 1. RC was present in the nucleus and the supernatant fractions of wild-type cells. Nuclear RC content was significantly ($P < 0.01$) increased in transfectants at 0, 24, or 48 h of culture; the densitometric data showed 286 ± 8.6, 263 ± 18.5, or 164 ± 10.1 (percentage of wild-type cells; means ± SEM for four experiments), respectively. After 48 h, the difference of nuclear RC content between wild-type cells and transfectants was less marked. This seemed to be time-dependent changes after serum withdrawal.

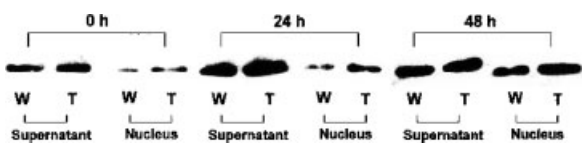

Fig. 1. Localization of RC in the nucleus of the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type; W) or stable RC/pCXN2-transfected cells (T). Cells (1×10^5) were cultured for 72 h in medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS. After medium change, cells were incubated for 24, 48, or 72 h, and the nucleus and the 1,000 g supernatant (containing mitochondria, microsomes, and cytoplasm) were prepared from the cell lysate. RC was analyzed by immunoblotting with an anti-RC antibody. The figure shows one of four experiments with separate samples. Densitometric data indicated means ± SEM for four experiments with separate cells.

Change in Gene Expression of Mineral Ion Transport-Related Proteins in Kidney NRK52E Cells Overexpressing Regucalcin

The change in ENaC, Na, K-ATPase, and ROMK mRNA expressions in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells with subconfluency is shown in Figure 2. Cells with subconfluency were cultured for 24 or 48 h in the absence of BS. Overexpression of RC caused a significant increase in ROMK mRNA expression with culture for 48 h, while it did not have a significant effect on ENaC, Na, K-ATPase, and G3PDH mRNA expressions with culture of 24 or 48 h.

The effect of aldosterone on the expression of ENaC, Na, K-ATPase, and ROMK mRNAs in wild-type cells is shown in Figure 3. Culture with aldosterone (10^{-8} or 10^{-7} M) for 24 h caused a significant increase in ENaC mRNA expression, while its expression for 48 h was not changed (Fig. 3A). The expression of Na, K-ATPase (Fig. 3B), or ROMK (Fig. 3C) mRNAs was significantly increased in the presence of aldosterone (10^{-8} or 10^{-7} M) for 24 or 48 h. G3PDH mRNA expression was not changed by aldosterone (Fig. 3D). The effect of aldosterone (10^{-8} M) in increasing ENaC, Na, K-ATPase, or ROMK mRNA expressions in wild-type cells cultured for 24 or 48 h was not seen in the transfectants (Fig. 4).

The effect of PTH on NaPi-IIa mRNA expression in wild-type cells with subconfluency is shown in Figure 5. The expression of NaPi-IIa mRNA was not significantly changed in transfectants (Fig. 5A). Culture with PTH (10^{-8} or 10^{-7} M) for 24 or 48 h caused a significant decrease in NaPi-IIa mRNA expression in wild-type cells (Fig. 5B). PTH did not have an effect on G3PDH mRNA expression in wild-type cells (Fig. 5C). The effect of PTH (10^{-8} M) in decreasing NaPi-IIa mRNA expression was also seen in transfectants (Fig. 5D).

Effect of Aldosterone or PTH on Angiotensinogen mRNA Expression in Kidney NRK52E Cells Overexpressing Regucalcin

The effect of aldosterone or PTH on angiotensinogen mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells is shown in Figure 6. Cells with subconfluency were cultured for 24 or 48 h in the

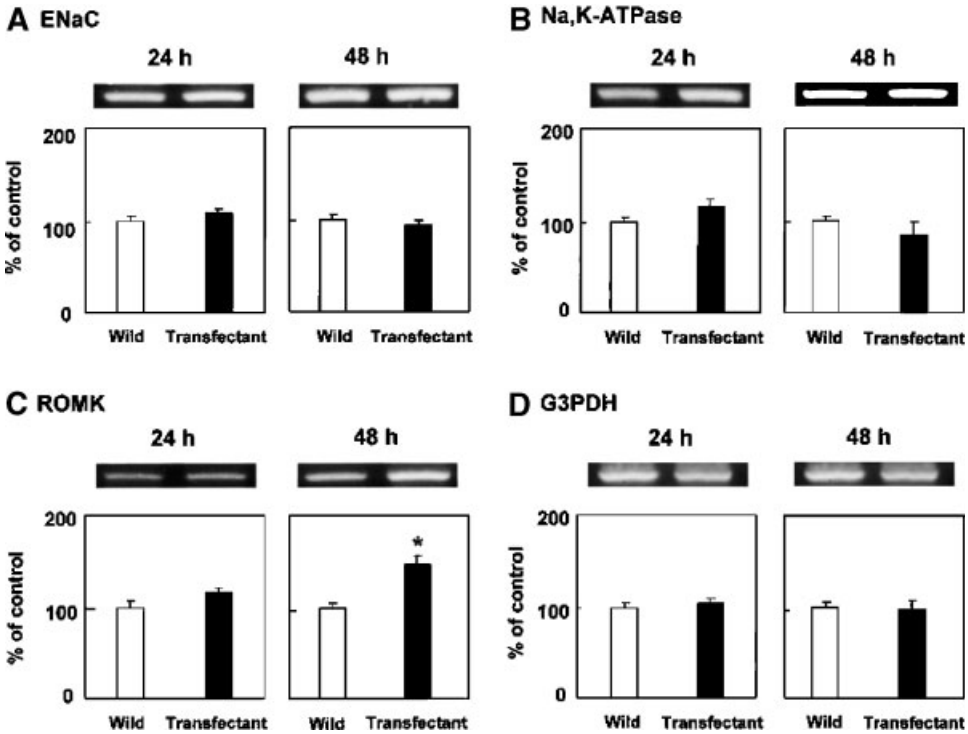

Fig. 2. Change in mRNA expression of various proteins that are related mineral transport in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in medium containing 5% BS to obtain subconfluent monolayers, and then exchanged to culture medium without BS. After medium change, 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 ENaC (**A**), Na, K-ATPase (**B**), ROMK (**C**), and G3PDH (**D**) cDNAs. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^*P < 0.01$ compared with the value obtained from wild-type cells.

absence of BS. The expression of angiotensinogen mRNA was not significantly changed in transfectants (Fig. 6A). Culture with aldosterone (10^{-8} M) for 24 or 48 h did not have a significant effect on angiotensinogen mRNA expression in wild-type cells and transfectants (Fig. 6B). Angiotensinogen mRNA expression was significantly decreased in wild-type cells cultured for 48 h in the presence of PTH (10^{-8} M) (Fig. 6C). These effects were also observed in transfectants (Fig. 6C).

Change in Gene Expression of Calcium Ion-Related Proteins in Kidney NRK52E Cells Overexpressing Regucalcin

The change in L-type Ca^{2+} channel, CaR, and Na^+-Ca^{2+} exchanger mRNA expressions in the cloned rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells is shown in Figure 7. Cells with subconfluency were cultured for 24 or 48 h in the presence of BS. The expression of L-type Ca^{2+} channel (Fig. 7A) and CaR (Fig. 7B) mRNAs was significantly decreased in transfectants cultured for 24 or 48 h. Na^+-Ca^{2+} exchanger mRNA expression was not significantly changed in transfectants (Fig. 7C).

The effect of PTH on L-type Ca^{2+} channel, CaR, and Na^+-Ca^{2+} exchanger mRNA expressions in wild-type cells is shown Figure 8. Cells with subconfluency were cultured for 24 or 48 h in the presence of PTH (10^{-8} or 10^{-7} M) without BS. The expression of L-type Ca^{2+} channel (Fig. 8A) and CaR (Fig. 8B) mRNAs was significantly decreased in wild-type cells cultured for 24 or 48 h in the presence of PTH (10^{-8} or 10^{-7} M). Na^+-Ca^{2+} exchanger mRNA expression in wild-type cells was significantly increased with culture for 48 h in the presence of PTH (10^{-7} M) (Fig. 8C).

The effect of PTH (10^{-8} M) in decreasing L-type Ca^{2+} channel and CaR mRNA expressions in wild-type cells was also seen in transfectants cultured for 24 or 48 h in the absence of BS (Fig. 9A,B). Na^+-Ca^{2+} exchanger mRNA expression was not significantly changed in transfectants cultured with PTH (10^{-8} M) for 24 or 48 h (Fig. 9C).

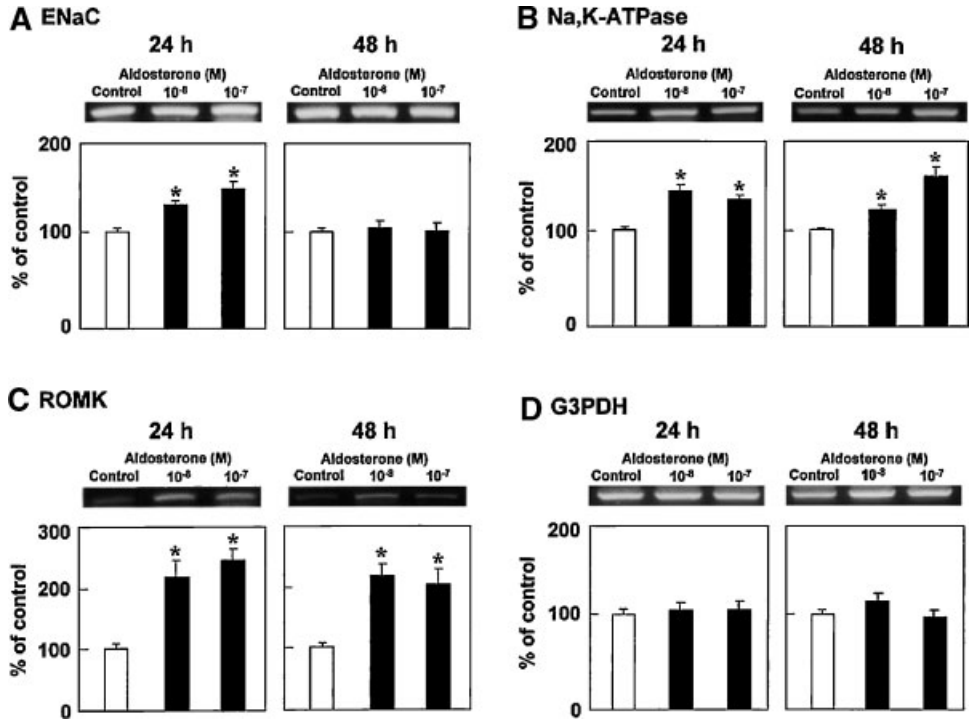

Fig. 3. Effect of aldosterone on mRNA expression of various proteins that are related mineral transport in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type). Cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle or aldosterone (10^{-8} or 10^{-7} M) without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for ENaC (**A**), Na, K-ATPase (**B**), ROMK (**C**), and G3PDH (**D**) cDNAs. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^*P < 0.01$ compared with the value obtained from wild-type cells.

DISCUSSION

RC is expressed in the cloned rat kidney proximal tubular epithelial NRK52E cells, and RC mRNA expression is stimulated with treatment of aldosterone or PTH in the cells [Nakagawa and Yamaguchi, 2005a]. Overexpression of RC has been shown to suppress cell proliferation and apoptotic cell death in NRK52E cells, and it modulates the gene expression of proteins which are related to cell proliferation and apoptosis [Nakagawa et al., 2005; Nakagawa and Yamaguchi, 2005b]. RC may play a regulatory role in kidney proximal tubular epithelial cells. This study demonstrates that overexpression of RC causes a remarkable increase in its nuclear localization, and that it has suppressive effects on the gene expression of L-type Ca^{2+} channel and CaR which regulate intracellular Ca^{2+} signaling in NRK52E cells, suggesting that RC plays a role in the regulation of transcriptional activity in the nucleus.

RC has been shown to translocate passively to the nucleus of rat liver, and it has been demonstrated to regulate nuclear function in regenerating rat liver and the cloned rat hepatoma H4-II-E cells with proliferative cells cultured without serum [Tsurusaki et al., 2000; Misawa et al., 2002; Tsurusaki and Yamaguchi, 2004]. Moreover, RC was found to be present in the nucleus of cloned normal rat kidney proximal tubular epithelial NRK52E cells. This localization was markedly enhanced in NRK52E cells overexpressing RC, suggesting that cytoplasma RC passively translocates in nucleus. RC may play a role in the regulation of nuclear function.

Overexpression of RC caused a significant increase in ROMK mRNA expression in NRK52E cells, while it did not have an effect on Na, K-ATPase, and ENaC mRNA expressions. Culture with aldosterone caused a significant increase in ENaC, Na, K-ATPase, and ROMK mRNA expressions in NRK52E cells (wild-type). The expression of these genes has been reported to increase by treatment with aldosterone in rat kidney cells [Wald et al., 1998; Masilamani et al., 1999; Vanessa et al.,

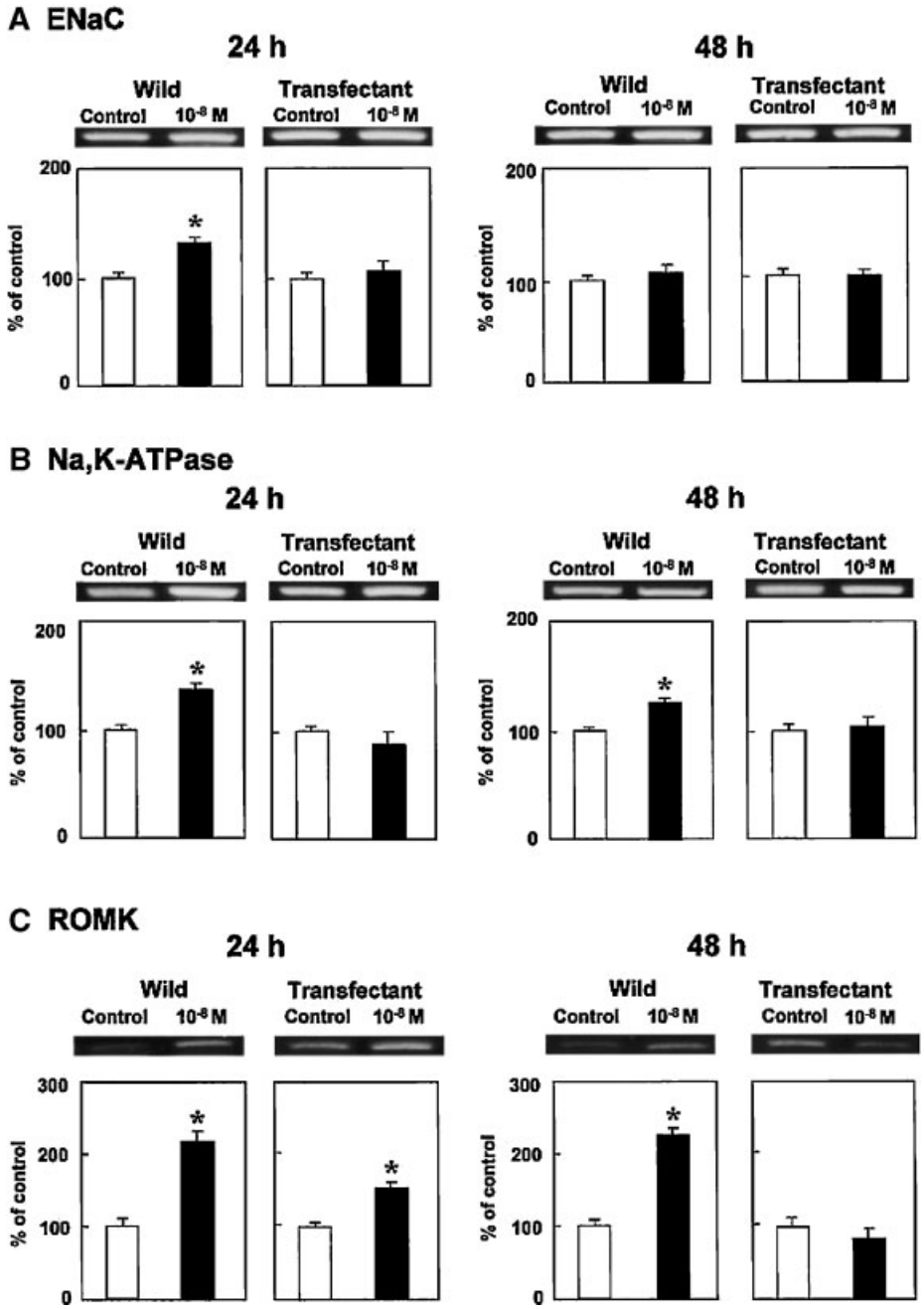

Fig. 4. Effect of aldosterone on mRNA expression of various proteins that are related mineral transport in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle or aldosterone (10^{-8} M) without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for ENaC (**A**), Na, K-ATPase (**B**), ROMK (**C**) cDNAs. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^*P < 0.01$ compared with the control (none) value.

2001]. The effect of aldosterone in increasing ENaC and Na, K-ATPase mRNA expressions was not observed in NRK52E cells overexpressing RC. However, overexpression of RC had a significant stimulatory effect on ROMK mRNA expression in the hormone-untreated transfectants. Culture with aldosterone did not significantly enhance ROMK mRNA expression in transfectants cultured for 48 h after serum withdrawal. Aldosterone can upregulate RC mRNA expression in NRK52E cells [Nakagawa and Yamaguchi, 2005a]. Presumably, RC partly

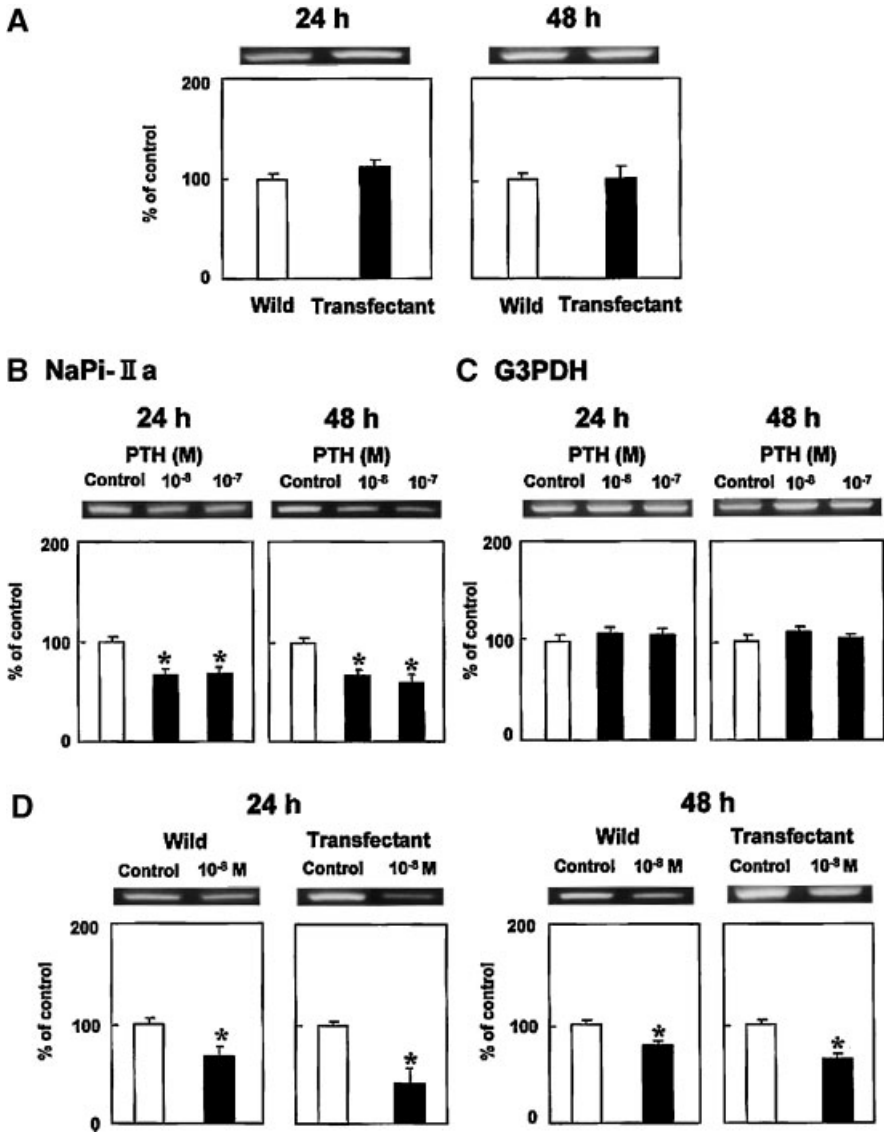

Fig. 5. Effect of PTH on NaPi-IIa mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle (**A**) or PTH (10^{-8} or 10^{-7} M) (**B–D**) without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^*P < 0.01$ compared with the control (none) value.

have regulatory effects on the aldosterone-induced increase in certain gene expressions in NRK52E cells.

The expression of NaPi-IIa and angiotensinogen mRNAs was not significantly changed in NRK52E cells overexpressing RC. Culture with PTH caused a significant decrease in NaPi-IIa and angiotensinogen mRNA expressions in wild-type cells. The hormonal effect on gene expression was observed in transfectants. Presumably, overexpression of RC does not have effects on NaPi-IIa and angiotensinogen mRNA expressions in kidney NRK52E cells.

Overexpression of RC was found to suppress markedly the expression of L-type Ca^{2+} channel or CaR mRNAs in NRK52E cells. The expression of Na^{+}-Ca^{2+} exchanger mRNA was not significantly changed in the transfectants. The blockade of calcium influx through L-type calcium channels has been shown to attenuate mitochondrial injury and apoptosis in hypoxia

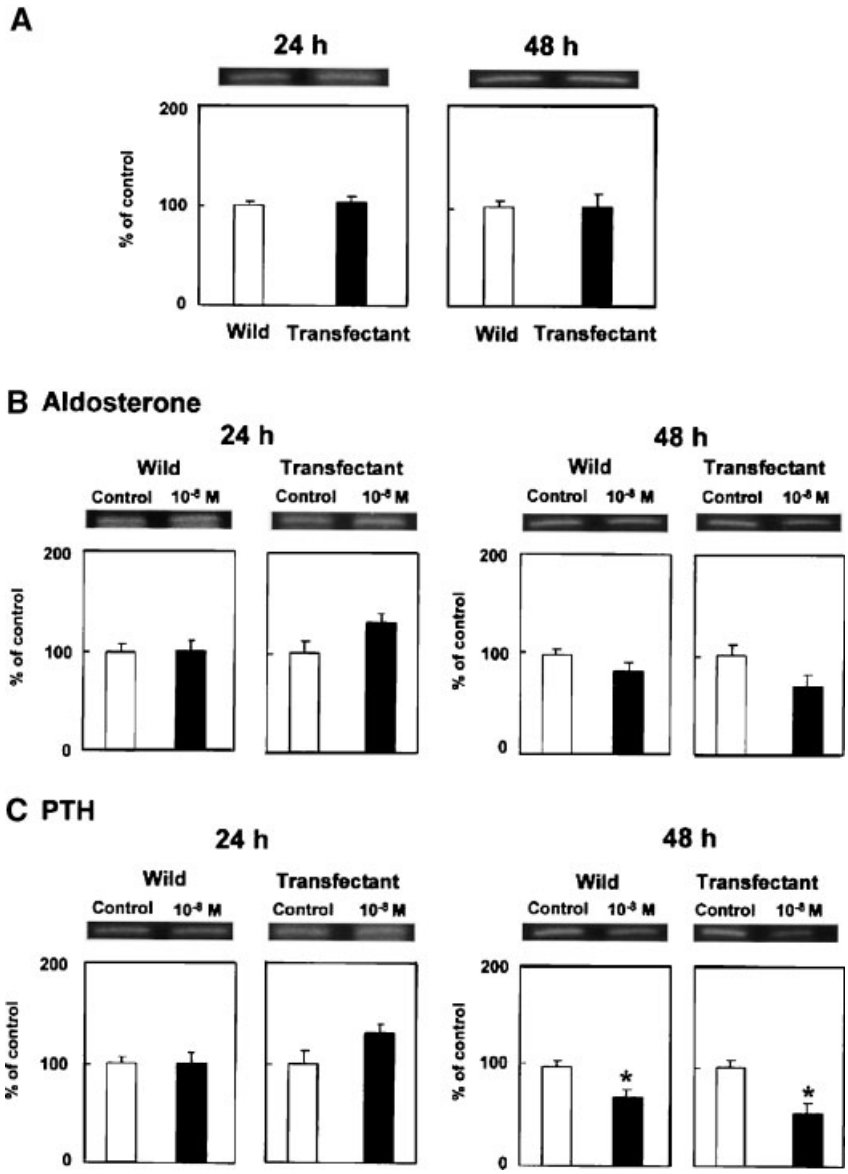

Fig. 6. Effect of aldosterone or PTH on angiotensinogen mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle (**A**), aldosterone (10^{-8} M) (**B**), or PTH (10^{-8} M) (**C**) without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^{*}P < 0.01$ compared with the control (none) value.

renal tubular cells [Tanaka et al., 2004]. The entry of calcium through L-type Ca^{2+} channels induces mitochondrial disruption and cell death [Cano-Abad et al., 2001]. CaR participates in the regulation of renal Ca^{2+} transport [Ba and Friedman, 2004]. It is speculated that RC regulates intracellular Ca^{2+}-signaling pathway through its suppressive effect on L-type Ca^{2+} channel or CaR mRNA expression in the kidney proximal tubular epithelial cells.

PTH may alter the expression of L-type Ca^{2+} channel or CaR mRNAs in kidney proximal tubular epithelial cells [Barry et al., 1998; Riccardi et al., 2000]. This study demonstrates that culture with PTH caused a significant decrease in L-type Ca^{2+} channel or CaR mRNA expressions in kidney NRK52E cells. PTH significantly increased Na^{+}-Ca^{2+} exchanger mRNA expression in NRK52E cells. PTH has been shown to activate Na^{+}-Ca^{2+} exchanger in renal

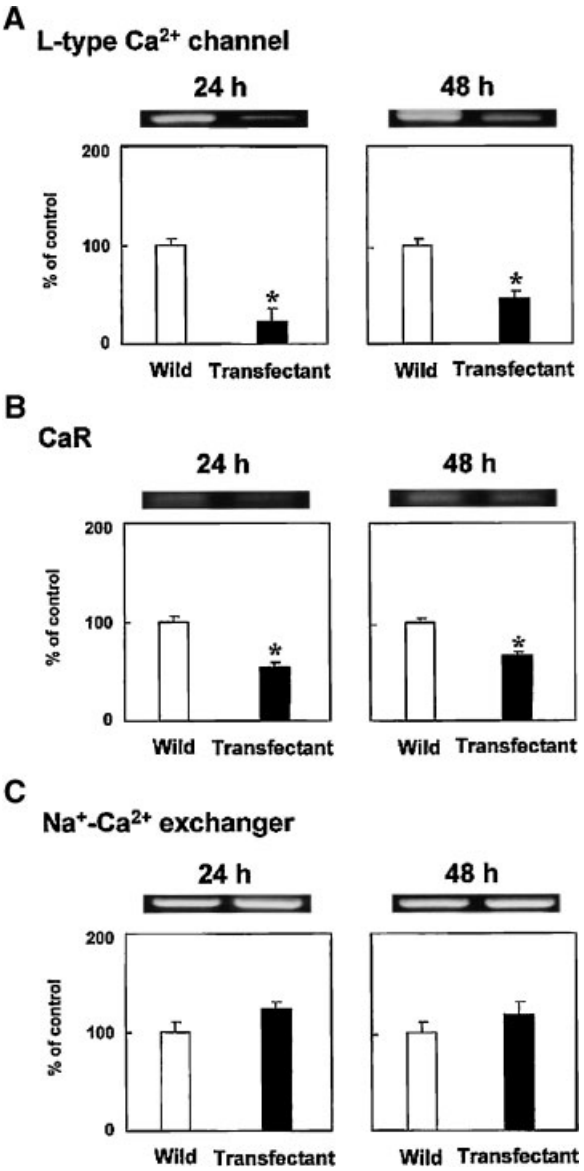

Fig. 7. Change in L-type Ca^{2+} channel or CaR mRNA expressions in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 48 h in medium without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for L-type Ca^{2+} channel (**A**), CaR (**B**), and Na^{+}-Ca^{2+} exchanger (**C**) cDNAs. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^{*}P < 0.01$ compared with the value obtained from wild-type cells.

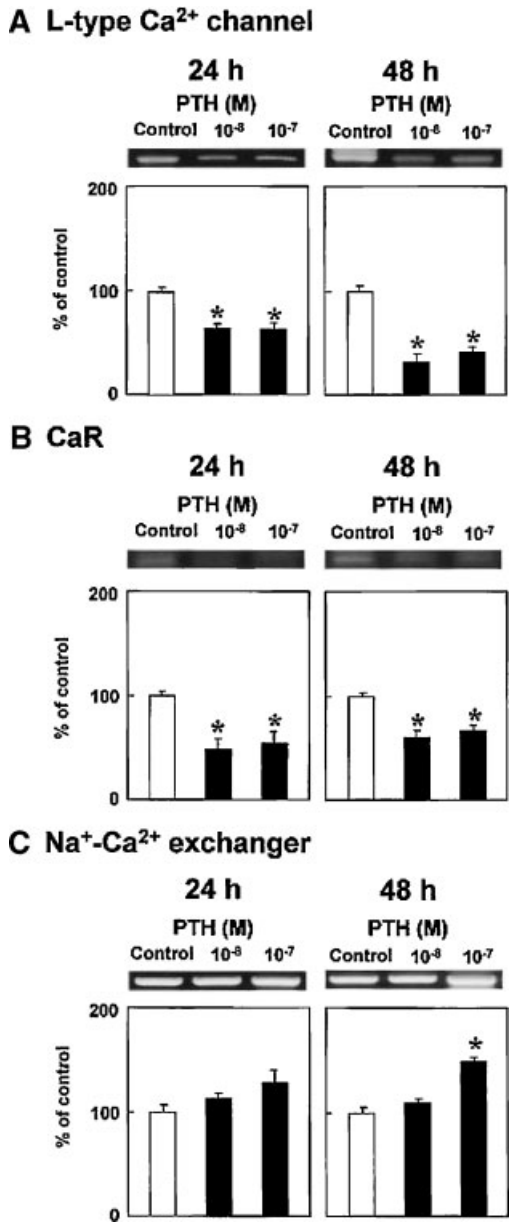

Fig. 8. Effect of PTH on L-type Ca^{2+} channel or CaR mRNA expressions in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type). Cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle or PTH (10^{-8} or 10^{-7} M) without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for L-type Ca^{2+} channel (**A**), CaR (**B**), and Na^{+}-Ca^{2+} exchanger (**C**) cDNAs. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). $^{*}P < 0.01$ compared with the control (none) value.

basolateral membrane vesicle [Scoble et al., 1985], suggesting that the hormone stimulates reabsorption of calcium in kidney proximal tubular epithelial cells. The effect of PTH on L-type Ca^{2+} channel, CaR, and Na^{+}-Ca^{2+} exchanger mRNA expressions was not significantly attenuated in NRK52E cells overexpressing RC. These observations may support the view that overexpression of RC does not regulate the effect of PTH on gene expression in NRK52E cells.

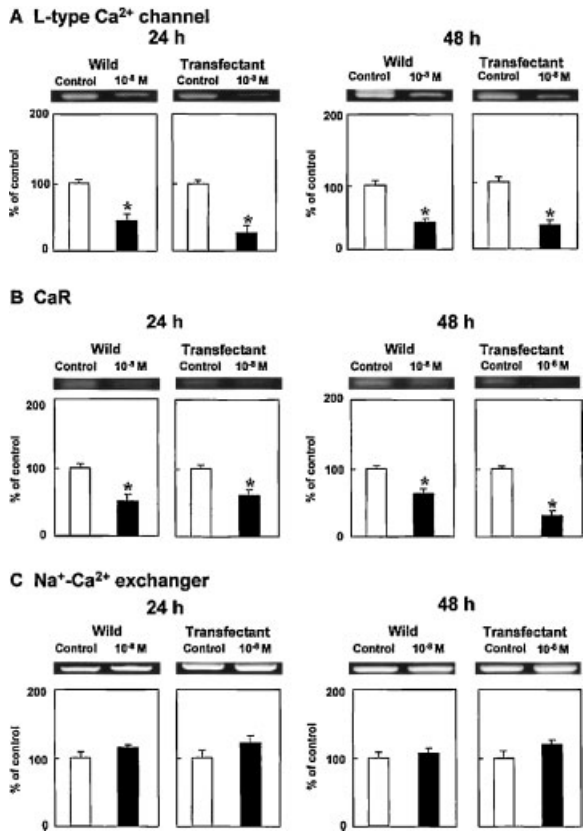

Fig. 9. Effect of PTH on L-type Ca^{2+} channel or CaR mRNA expressions in the cloned normal rat kidney proximal tubular epithelial NRK52E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle or PTH (10^{-8} M) without BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers for L-type Ca^{2+} channel (**A**), CaR (**B**), and Na^{+}-Ca^{2+} exchanger (**C**) cDNAs. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as percentage of control (mean ± SEM of five experiments with separate cells). *$P < 0.01$ compared with the control (none) value.

The expression of RC mRNA in NRK52E cells has been shown to be enhanced by the treatment of PTH [Nakagawa and Yamaguchi, 2005a], suggesting that RC partly mediates cellular response for PTH in kidney cells. Overexpression of RC did not attenuate the expression of L-type Ca^{2+} channel or CaR mRNAs, which is decreased by PTH treatment, in NRK52E cells. RC could decrease L-type Ca^{2+} channel or CaR mRNA expressions in NRK52E cells. From these observations, it is assumed that RC partly contributes as a mediator in cellular response for stimulation of PTH in NRK52E cells.

Whether calcium handling in NRK52E cells is changed in transfectants overexpressing RC is unknown. It cannot exclude the possibility that the increase in intracellular calcium may have effects on gene expression in transfectants. However, overexpression of RC has been shown to have suppressive effects on apoptosis caused with culture of Bay K 8644, a stimulator of calcium entry into cells, in NRK52E cells without serum [Nakagawa and Yamaguchi, 2005b]. Presumably, the effects of RC on gene expression are not mediated through change in calcium handling in transfectants.

RC has been shown to play a role in the regulation of intracellular Ca^{2+} transport; the protein activates Ca^{2+}-pumping enzymes (Ca^{2+}-ATPase) in the basolateral membranes, mitochondria, and microsomes in rat kidney cortex [Kurota and Yamaguchi, 1997a,b; Xue et al., 2000]. RC may regulate intracellular Ca^{2+} homeostasis in kidney proximal tubular epithelial cells so that Ca^{2+} is passed through transcellular transport. Moreover, RC was found to suppress the expression of L-type Ca^{2+} channel or CaR mRNAs in NRK52E cells. This finding support the view that RC plays a physiologic role in the regulation of intracellular Ca^{2+} homeostasis in kidney proximal tubular epithelial cells.

In conclusion, this study demonstrates that overexpression of RC causes a remarkable increase in its nuclear localization, and that it has suppressive effects on the gene expression of L-type Ca^{2+} channel or CaR which regulates intracellular Ca^{2+} signaling in the cloned normal rat kidney proximal tubular epithelial NRK52E cells.

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