Journal of Cellular Biochemistry 95:589–597 (2005)

Hormonal Regulation on Regucalcin mRNA Expression in Cloned Normal Rat Kidney Proximal Tubular Epithelial NRK52E Cells

Taeko Nakagawa and Masayoshi Yamaguchi*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

Abstract Regucalcin is a regulatory protein in cell signaling. This study was undertaken to determine whether regucalcin mRNA expresses in the cloned normal rat kidney proximal tubular epithelial NRK52E cells and its expression regulates due to hormones and cell signaling-related factors. Cells with subconfluency were cultured for 24, 48, or 72 h in a Dulbecco's modified Eagle medium supplemented with non-essential amino acid without bovine serum (BS). The result of Western blot analysis showed that regucalcin protein was present in the NRK52E cells. The expression of regucalcin mRNA in the cells was determined using reverse transcription-polymerase chain reaction (RT-PCR). Regucalcin mRNA expression in the NRK52E cells was significantly increased by culture with parathyroid hormone (PTH, 10^{-8} or 10^{-7} M), aldosterone (10^{-8} or 10^{-7} M), or dexamethasone (10^{-8} M). The presence of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, 10^{-8} or 10^{-7} M) or calcitonin (10^{-9} or 10^{-8} M) did not have a significant effect on regucalcin mRNA levels in the cells. Culture with dibutyryl cyclic AMP (DcAMP, 10^{-5} or 10^{-4} M) or phorbol 12-myristate 13-acetate (PMA, 10^{-6} M), an activator of protein kinase C, caused a significant increase in regucalcin mRNA expression. The presence of staurosporine (10^{-8} M) caused a significant decrease in regucalcin mRNA expression. Dibucaine (10^{-7} M), PD98059 (10^{-7} M), or vanadate (10^{-6} or 10^{-5} M) did not have an effect on regucalcin mRNA levels. The present study demonstrates that regucalcin mRNA and its protein are expressed in the cloned normal rat kidney proximal tubular epithelial NRK52E cells, and that the expression is enhanced by hormones which regulate ion transport in the proximal tubule. J. Cell. Biochem. 95: 589–597, 2005.

Key words: regucalcin; parathyroid hormone; aldosterone; calcium; cyclic AMP; protein kinase C; kidney cell; NRK52E cell

Regucalcin was found as a novel Ca^{2+}-binding protein not including the EF-hand motif [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1992; Shimokawa and Yamaguchi, 1993]. The gene of regucalcin is highly conserved in vertebrate species [Misawa and Yamaguchi, 2000]. The human and rat regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. Regucalcin is greatly expressed in liver and kidney cortex, and the expression is mediated through Ca^{2+}-signaling mechanism due to hormonal stimulation [Yamaguchi and Isogai, 1993; Murata and Yamaguchi, 1999]. AP-1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1998; Misawa and Yamaguchi, 2002].

Recent study has demonstrated that regucalcin plays a multifunctional role as a regulatory protein on intracellular signaling process in the cytoplasm and nucleus of cells [Yamaguchi, 2000a,b in reviews]. 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, nitric oxide synthase, and in the suppression of nuclear DNA and RNA syntheses [Yamaguchi, 2000a,b, 2005]. Regucalcin has also been demonstrated to have a suppressive effect on cell proliferation, cell death, and apoptosis induced by various factors [Misawa et al., 2002; Izumi and Yamaguchi, 2004]. Moreover,

*Correspondence to: Masayoshi Yamaguchi, PhD, Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan.
E-mail: yamaguch@u-shizuoka-ken.ac.jp

Received 18 October 2004; Accepted 14 December 2004
DOI 10.1002/jcb.20422

regucalcin has been shown to modulate tumor-related gene expression by binding to protein and DNA in the nucleus of cloned rat hepatoma H4-II-E cells [Tsurusaki et al., 2000; Tsurusaki and Yamaguchi, 2003, 2004]. Regucalcin has been proposed to play a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation [Yamaguchi, 2005 in review].

Regucalcin is greatly localized in liver, and its relatively higher levels are also found in kidney cortex but not the medulla of rats [Shimokawa and Yamaguchi, 1992; Yamaguchi and Kurota, 1995]. Regucalcin has been shown to play a role in the regulation of intracellular Ca^{2+} homeostasis [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 nitric oxide synthase [Ma and Yamaguchi, 2003] in the cytosol and nucleus of rat kidney cortex. Thus, regucalcin has been shown to play an important role in the regulation of cell function in kidney cortex.

This study, furthermore, was undertaken to determine the expression of regucalcin mRNA in the cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro. The role of regucalcin in kidney cells in vitro has not been yet clarified. We found that regucalcin mRNA expression in the NRK52E cells is enhanced by stimulation of hormones that are related to ion transport in the kidney proximal tubular epithelial cells in vitro.

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)), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), D-aldosterone, dexamethasone, dibutyryl cyclic AMP (DcAMP), phorbol 12-myristate 13-acetate (PMA), dibucaine, PD98059, and staurosporine were purchased from Sigma (St. Louis, MO). S(−)-Bay K 8644 was obtained from Research Biochemicals International (Natick, MA). Calcitonin (CT; synthetic [$Asu^{1,7}$] eel calcitonin) was supplied through the courtesy of Asahi Chemical Industry Co., Ltd. (Shizuoka, Japan). Vanadate and other chemicals were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

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 a DMEM supplemented with 5% BS, non-essential amino acid, and 50 U/ml 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 various factors. Cells were washed three times with PBS after culture. The polled cells were 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 5,500*g* in a refrigerated centrifuge for 5 min to remove nuclei and mitochondria. The 5,500*g* supernatant was pooled to analyze regucalcin protein by Western blot. Protein concentration in the 5,500*g* supernatant of cell homogenate was determined by the method of Lowry et al. [1951].

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 Hemacytometer plate. For each dish, we took the average of two countings.

Western Blot Analysis

The homogenate from the cloned normal rat kidney proximal tubular epithelial NRK52E cells cultured with 5% BS was centrifuged for 10 min at 5,500*g* at 4°C, and the supernatant was 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 polyviny-

lidene difluoride membrane at 100 mA for 3 h. The membranes were incubated with a polyclonal rabbit anti-regucalcin 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 horse-radish 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 manufacture'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]. Rat kidney proximal tubular epithelial cells were homogenized in buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sascosyl, 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.

RT-PCR Analysis

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 of regucalcin or glyceroaldehyde-3-phosphate dehydrogenase (G3PDH). The primers generated based on the published rat sequences. Primers for regucalcin cDNA were 5′-(618)-AGATGAACAAATCCCAGAT-(636)-3′ and 5′-(924)-TCACCCTGCATAGGAATAT-(906)-3′ [Misawa and Yamaguchi, 2000]. Primers for G3PDH cDNA were 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense strand) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense strand) from the G3PDH Amplimer Set (Clontech, Palo Alto, CA). RT-PCR was performed by 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

Expression of Regucalcin in Cloned Kidney Proximal Tubular Epithelial Cells

The cloned normal rat kidney proximal tubular epithelial NRK52E cells were cultured for 72 h to reach subconfluent monolayers in the presence of 5% BS. The results of RT-PCR analysis showed that regucalcin mRNA was expressed in the cloned normal rat kidney proximal tubular epithelial cells (Fig. 1A). Also,

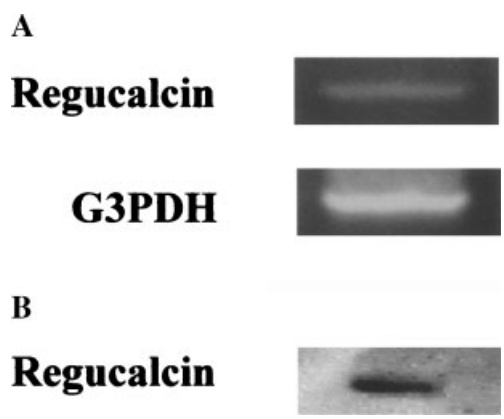

Fig. 1. Expression of regucalcin in the cloned normal rat kidney proximal tubular epithelial NRK5 cells. Cells were cultured for 72 h in the presence of 5% bovine serum (BS). Total RNAs (2 μg) extracted from the cells were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers (**A**). Western blot analysis was carried out on the extracts (10 μg of the 5,500*g* supernatant of cell homogenate) obtained from the NRK52E cells (**B**). The figure shows one of four experiments with separate samples.

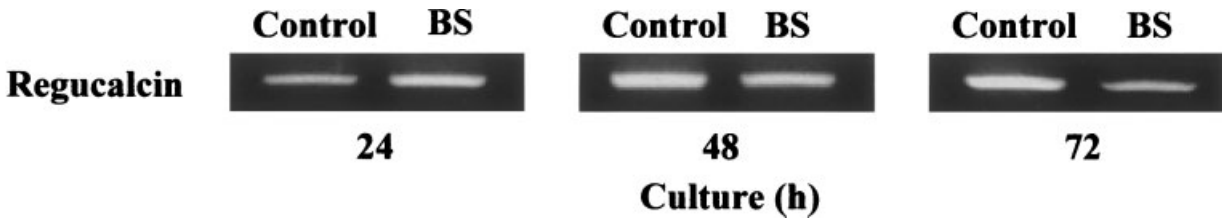

Fig. 2. Effect of BS on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cells were cultured for 72 h in the presence of 5% BS. Cells with subconfluency were cultured for 24, 48, or 72 h in the presence or absence of 5% BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for regucalcin mRNA expression in the cells cultured for 24, 48, or 72 h in the presence of BS were 144 ± 5.0, 69 ± 4.1, or 45 ± 6.9 (% of control; mean ± SEM for four experiments), respectively. The increase in regucalcin mRNA levels seen at 24 h, or its decrease observed at 48 or 72 h was significant ($P < 0.01$) as compared with that of control levels.

Western blot analysis showed that regucalcin protein was present in the NRK52E cells (Fig. 1B). Moreover, the cells with subconfluency were cultured for 24, 48, or 72 h in the presence or absence of 5% BS. The expression of regucalcin mRNA in the NRK52E cells was significantly increased by 24-h culture with 5% BS (Fig. 2). However, its expression was significantly decreased by 48- or 72-h culture with 5% BS (Fig. 2). These results suggest that regucalcin mRNA expression is regulated by various factors (including hormones and cytokines) that are present in BS.

Effect of Hormones on Regucalcin mRNA Expression in Cloned Kidney Proximal Tubular Epithelial Cells

The cloned normal rat kidney proximal tubular epithelial NRK52E cells were cultured for 72 h in the presence of 5% BS. The cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, PTH (10^{-8} or 10^{-7} M), $1,25(OH)_2D_3$ (10^{-8} or 10^{-7} M), CT (10^{-9} or 10^{-8} M), aldosterone (10^{-8} or 10^{-7} M), or dexamethasone (10^{-8} or 10^{-7} M) in the absence of BS. Culture with PTH (10^{-8} or 10^{-7} M) for

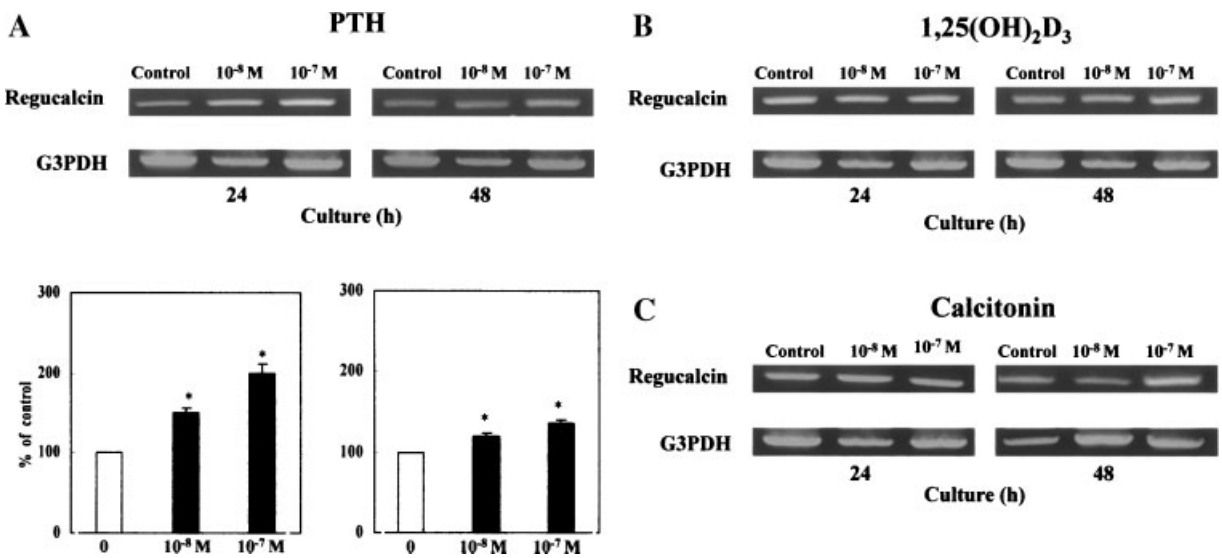

Fig. 3. Effect of parathyroid hormone (PTH) (**A**), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (**B**), or calcitonin (**C**) on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. After culture for 72 h with 5% BS, cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, PTH (10^{-8} or 10^{-7} M), $1,25(OH)_2D_3$ (10^{-8} or 10^{-7} M), or CT (10^{-9} or 10^{-8} M) in the absence of BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for regucalcin mRNA levels in the cells cultured for 24 or 48 h in the presence of hormone were indicated as % of control value (mean ± SEM for four experiments). $^*P < 0.01$, compared with the control value.

24 or 48 h caused a significant increase in regucalcin mRNA levels in the NRK52E cells (Fig. 3A). The presence of $1,25(OH)_2D_3$ (10^{-8} or 10^{-7} M) or CT (10^{-9} or 10^{-8} M) did not have a significant effect on regucalcin mRNA expression in the cells (Fig. 3B,C).

The expression of regucalcin mRNA was significantly increased by 24-h culture in the presence of aldosterone (10^{-7} M) (Fig. 4A). This expression was further enhanced by 48-h culture with aldosterone (10^{-8} or 10^{-7} M) (Fig. 4A). Meanwhile, culture with dexamethasone (10^{-8} or 10^{-7} M) for 24 h did not cause a significant alteration in regucalcin mRNA levels in the NRK52E cells (Fig. 4B). Regucalcin mRNA expression was significantly enhanced by 48-h culture with 10^{-8} M dexamethasone (Fig. 4B).

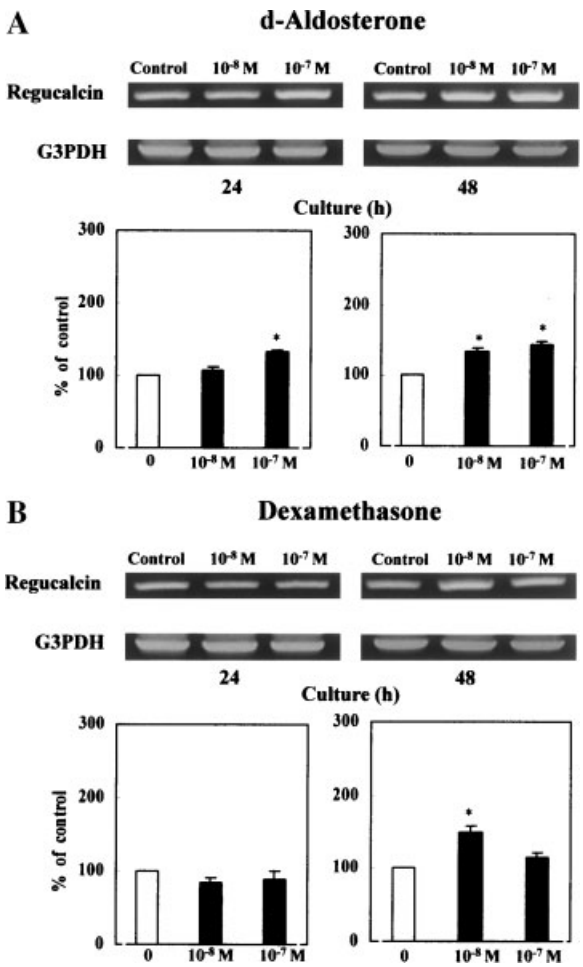

Fig. 4. Effect of aldosterone (**A**) or dexamethasone (**B**) on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, aldosterone (10^{-8} or 10^{-7} M), or dexamethasone (10^{-8} or 10^{-7} M) in the absence of BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for regucalcin mRNA levels in the cells cultured for 24 or 48 h in the presence of hormone were indicated as % of control (mean ± SEM for four experiments). $^*P < 0.01$, compared with the control value.

G3PDH mRNA expression in the NRK52E cells was not significantly changed by culture with PTH, $1,25(OH)_2D_3$, CT, aldosterone, or dexamethasone for 24 or 48 h (data not shown in Figs. 3 and 4).

Effect of Cell Signaling-Related Factors on Regucalcin mRNA Expression in Cloned Kidney Proximal Tubular Epithelial Cells

The cloned normal rat kidney proximal tubular epithelial NRK52E cells with subconfluency were cultured for 24, 48, or 72 h in a medium containing either vehicle, Bay K 8644 (2.5×10^{-6} M), DcAMP (10^{-5} or 10^{-4} M), or PMA (10^{-6} M) in the absence of BS. The presence of Bay K 8644 did not cause a significant alteration in regucalcin mRNA levels in the NRK52E cells (Fig. 5). Meanwhile, regucalcin mRNA expression was significantly increased by culture with DcAMP (10^{-5} or 10^{-4} M) or PMA (10^{-6} M) for 24 or 48 h (Fig. 6). G3PDH mRNA expression was not significantly changed in the presence of Bay K 8644, DcAMP or PMA for 24 or 48 h (Figs. 5 and 6).

Effect of Protein Kinase and Phosphatase Inhibitors on Regucalcin mRNA Expression in Cloned Kidney Proximal Tubular Epithelial Cells

The cloned normal rat kidney proximal tubular epithelial NRK52E cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, dibucaine (10^{-7} M), PD98059 (10^{-7} M), staurosporine (10^{-8} M), or vanadate (10^{-6} or 10^{-5} M) with an effective concentration for all function in the absence of BS. Regucalcin mRNA expression in the NRK52E cells was not significantly altered in the presence of dibucaine for 24 or 48 h (Fig. 7). Culture with PD98059 for 48 h caused a significant decrease in regucalcin mRNA levels in the cells (Fig. 7B). Staurosporine had a remarkable suppressive effect on regucalcin mRNA expression in the cells cultured for 24 or 48 h (Fig. 7A,B). Vanadate (10^{-6} or 10^{-5} M) did not cause a significant change on regucalcin mRNA levels (Fig. 8). Meanwhile, G3PDH mRNA expression was not significantly altered by culture with dibucaine, PD98059, staurosporine or vanadate (Figs. 7 and 8).

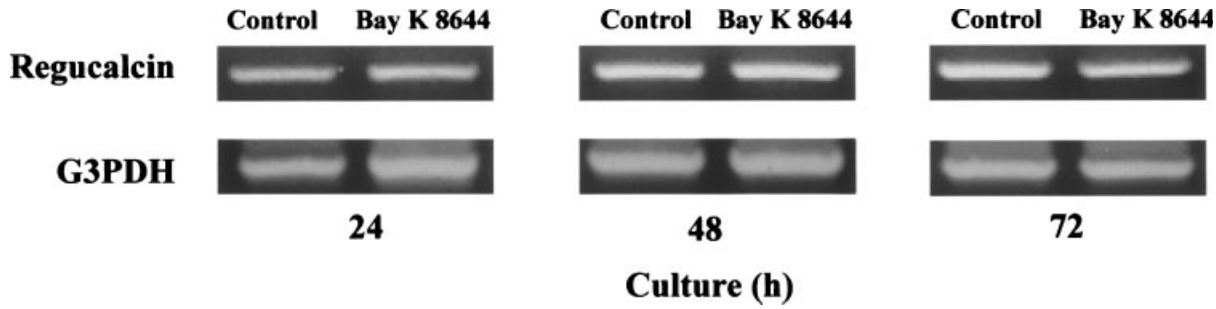

Fig. 5. Effect of Bay K 8644 on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cells with subconfluency were cultured for 24, 48, or 72 h in a medium containing either vehicle or Bay K 8644 (2.5 ± 10^{-6} M) in the absence of BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for regucalcin mRNA levels in the cells cultured for 24, 48, or 72 h in the presence of Bay K 8644 were 120 ± 5.8, 98 ± 6.2, or 83 ± 7.5 (% of control; mean ± SEM for four experiments), respectively. The data were not significant as compared with that of control levels.

DISCUSSION

It has been shown that regucalcin mRNA expression is stimulated in the kidney cortex of rats received a single intraperitoneal administration of calcium chloride, and that its expression is suppressed in thyroparathyroidectomized rats in vivo [Yamaguchi and Kurota, 1995]. The specific nuclear factor has been demonstrated to bind to the nuclear factor 1 (NF1) consensus motif TTGGC$(N)_6$CC in the promoter region of regucalcin gene in the kidney cortex of rats [Misawa and Yamaguchi, 2001]. The characterization of regucalcin mRNA expression in kidney cells, however, has not been determined yet. The present study finds that regucalcin mRNA is expressed in the cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro, and that its expression is enhanced by hormonal stimulation.

Calcium-regulating hormones including PTH, $1,25(OH)_2D_3$, or CT play a role in the regulation of calcium transport in kidney proximal tubular epithelial cells [Ng et al., 1982; Van Os, 1987]. Of these hormones, PTH has been found to stimulate the expression of regucalcin mRNA in the NRK52E cells. Regucalcin protein level significantly increased in the presence of PTH (data not shown). PTH has a stimulatory effect on the reabsorption of calcium in kidney proximal tubule [Agus et al., 1997]. Regucalcin has been shown to play a cell physiologic role as an activator in the ATP-dependent Ca^{2+} pumps in the basolateral

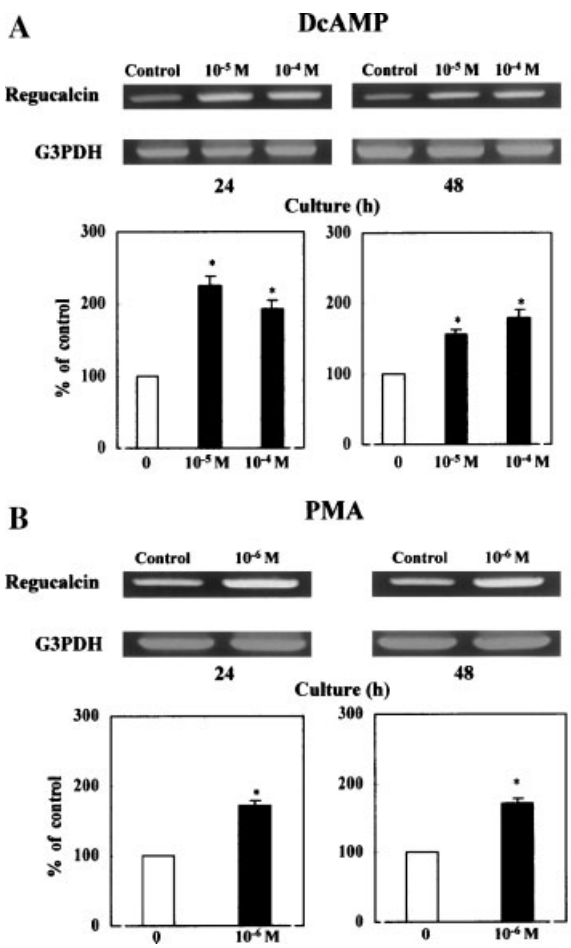

Fig. 6. Effect of dibutyryl cyclic AMP (DcAMP; **A**) or phorbol 12-myristate 13-acetate (PMA; **B**) on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, DcAMP (10^{-5} or 10^{-4} M) or PMA (10^{-6} M) in the absence of BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for regucalcin mRNA levels in the cells cultured for 24 or 48 h in the presence of DcAMP or PMA were indicated as % of control value (mean ± SEM for four experiments). $^*P < 0.01$, compared with the control value.

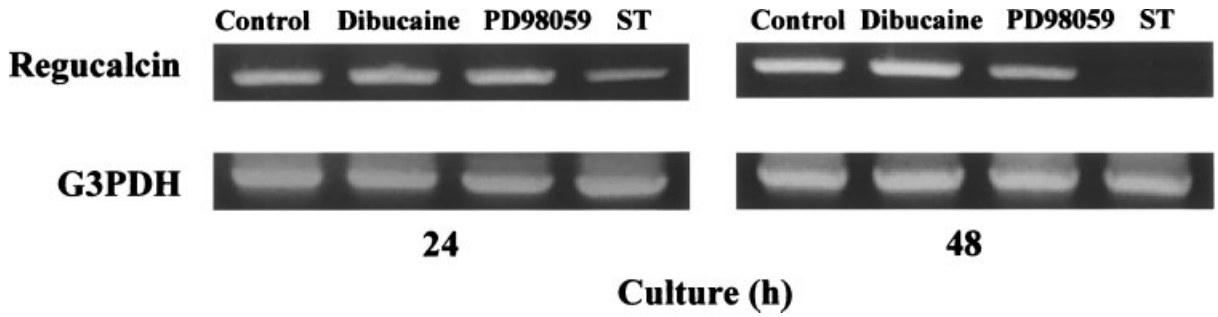

Fig. 7. Effect of dibucaine, PD98059, or staurosporine (ST) on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, dibucaine (10^{-7} M), PD98059 (10^{-7} M), or staurosporine (ST; 10^{-8} M) in the absence of BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows are one of four experiments with separate samples. The densitometric data for regucalcin mRNA levels in the cells cultured for 24 or 48 h in the presence of dibucaine, PD98059, or staurosporine (ST) were 103 ± 4.7, 97 ± 3.9, or 52 ± 6.7 with 24-h culture, and 115 ± 5.8, 80 ± 7.8 or 4 ± 2.0 with 48-h culture, respectively (mean ± SEM for four experiments). The decrease for 24- or 48-h culture with staurosporne was significant ($P < 0.01$) as compared with the control value.

membranes of rat kidney cortex [Kurota and Yamaguchi, 1997a,b,c]. It is speculated that regucalcin has a regulatory role in the reabsorption of calcium in kidney proximal tubule.

The expression of regucalcin mRNA in the cloned normal rat kidney proximal tubular epithelial NRK52E cells was enhanced by aldosterone, which has a stimulatory effect on the reabsorption of sodium in kidney proximal tuble. We also recognized that regucalcin protein level was also increased by aldosterone (data not shown). The mechanism by which aldosterone stimulates regucalcin mRNA expression in the NRK52E cells is unknown, at present It is speculated, however, that the expression may be partly mediated through the nuclear receptor for aldosterone in the cells. The ATP-dependent Ca^{2+} pump and the Na^{+}/Ca^{2+} exchange system are constituted in the basolateral membranes from rat kidney cortex [Van Heeswijk et al., 1984]. It is possible that the regulatory effect of aldosterone in kidney proximal tubule is partly mediated through the expression of regucalcin.

The expression of regucalcin mRNA in the NRK52E cells was significantly stimulated by culture with dexamethasone (10^{-8} M) for 48 h, although the concentration of 10^{-7} M did not have a significant effect on the expression. The lower concentration of dexamethasone may have an effect on regucalcin mRNA expression in the NRK52E cells. If dexamethasone enhances regucalcin mRNA in the NRK52E cells, the effect of dexamethasone in kidney proximal tubule may be partly mediated through regucalcin.

Bay K 8644, an agonist of calcium entry, caused a slight increase in regucalcin mRNA

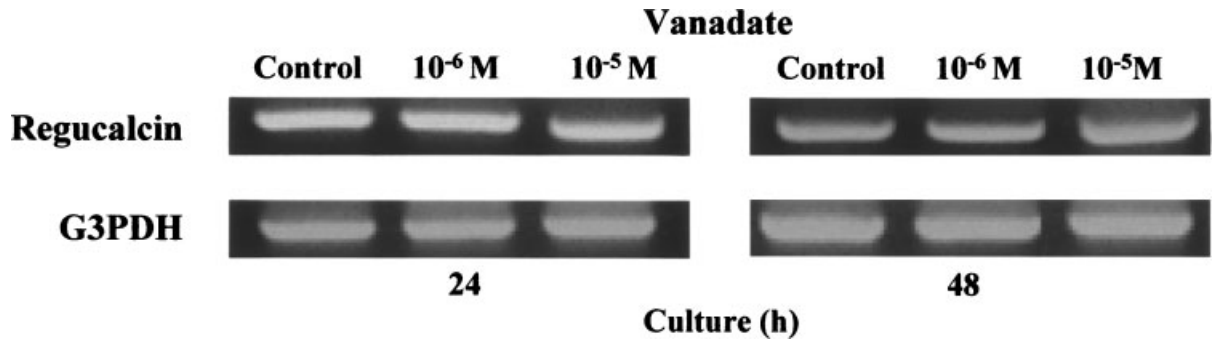

Fig. 8. Effect of vanadate on regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle or vanadate (10^{-6} or 10^{-5} M) in the absence of BS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for regucalcin mRNA levels in the cells were 107 ± 5.2 or 107 ± 4.6 with 24-h culture of vanadate (10^{-6} or 10^{-5} M), and 85 ± 8.2 or 112 ± 6.8 with 48-h culture of vanadate (10^{-6} or 10^{-5} M), respectively. Data were not significant as compared with the control value.

expression, although it is not significant. It can not exclude the possibility that an increase in intracellular calcium level provokes regucalcin mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells.

Regucalcin mRNA expression was found to be stimulated by DcAMP or PMA in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. PMA has an activatory effect on protein kinase C [Berridge, 1984]. DcAMP may have a role as second messenger [Rasmussen, 1970]. The expression of regucalcin mRNA in the 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 (IP_3)-released Ca^{2+} and protein kinase C in cells [Verheijen and Defize, 1997]. It is assumed that the effect of PTH in stimulating regucalcin mRNA expression in the NRK52E cells may be mediated through cyclic AMP and/or Ca^{2+}-dependent protein kinase C in the NRK52E cells.

Dibucaine is an antagonist of Ca^{2+}/calmodulin-dependent protein kinase [Vincenzi, 1982], and PD98059 is a ERK inhibitor [Peleck et al., 1993]. Regucalcin mRNA expression was not significantly inhibited in the presence of dibucaine or PD98059 in the NRK52E cells, suggesting that its expression is not mediated through Ca^{2+}/calmodulin-dependent protein kinase or protein tyrosine kinase. Meanwhile, regucalcin mRNA expression in the NRK52E cells was found to suppressed by staurosporine, an inhibitor of protein kinase C [Tamaoki et al., 1986]. This result may support the view that regucalcin mRNA expression is partly mediated through cell signaling which is related to protein kinase C in the NRK52E cells.

Vanadate is an inhibitor of protein tyrosine phosphatase [Hunter, 1995]. Culture with vanadate did not cause a significant alteration in regucalcin mRNA expression in the NRK52E cells. The expression of regucalcin mRNA may be involved in cell signaling which is involved in protein tyrosine phosphatase in the NRK52E cells.

The expression of regucalcin mRNA in the kidney proximal tubular NRK52E cells has been shown to stimulate by PTH or aldosterone in this study. In liver cells that express greatly regucalcin, whether PTH or aldosterone enhances regucalcin mRNA expression is unknown. Kidney is a target organ for PTH or aldosterone that regulates kidney function. Presumably, regucalcin plays a physiologic role in the cellular regulation of PTH or aldosterone.

In conclusion, it has been demonstrated that regucalcin mRNA expression is stimulated by addition of PTH or aldosterone in the cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro.

REFERENCES

Agus ZS, Chiu PJS, Goldberg M. 1997. Regulation of urinary calcium excretion in the rat. Am J Physiol 232: F545–F549.

Berridge MJ. 1984. Inositol trisphosphate and diacylglycerol as second messengers. Biochem J 220:345–360.

Chomczyshi P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159.

Hunter T. 1995. Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. Cell 80:225–236.

Izumi T, Yamaguchi M. 2004. Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-α or thapsigargin. J Cell Biochem 92:296–306.

Kurota H, Yamaguchi M. 1997a. Activatory effect of calcium-binding protein regucalcin on ATP-dependent calcium transport in the basolateral membranes of rat kidney cortex. Mol Cell Biochem 169:149–156.

Kurota H, Yamaguchi M. 1997b. Regucalcin increases Ca^{2+}-ATPase activity and ATP-dependent calcium uptake in the microsomes of rat kidney cortex. Mol Cell Biochem 177:201–207.

Kurota H, Yamaguchi M. 1997c. Inhibitory effect of regucalcin on Ca^{2+}/calmodulin-dependent protein kinase activity in rat renal cortex cytosol. Mol Cell Biochem 177: 239–243.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the hand of bactgeriophage T_4. Nature 224:680–685.

Lowry OH, Rosebrough MJ, Farr AJ, Randall FJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–273.

Ma ZJ, Yamaguchi M. 2003. Regulatory effect of regucalcin on nitric oxide synthase activity in rat kidney cortex cytosol: Role of endogenous regucalcin in transgenic rats. Int J Mol Med 12:201–206.

Misawa H, Yamaguchi M. 2000. The gene of Ca^{2+}-binding protein regucalcin is highly conserved in vertebrate species. Int J Mol Med 6:191–196.

Misawa H, Yamaguchi M. 2001. Involvement of nuclear factor-1 (NF1) binding motif in the regucalcin gene expression of rat kidney cortex: The expression is suppressed by cisplatin administration. Mol Cell Biochem 219:29–37.

Misawa H, Yamaguchi M. 2002. Identification of transcription factor in the promoter region of rat regucalcin gene: Binding of nuclear factor I-A1 to TTGGC motif. J Cell Biochem 84:795–802.

Misawa H, Inagaki H, Yamaguchi M. 2002. Suppression of cell proliferation and deoxyribonucleic acid synthesis in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84:143–149.

Morooka Y, Yamaguchi M. 2002. Endogenous regucalcin suppresses the enhancement of protein phosphatase activity in the cytosol and nucleus of kidney cortex in calcium-administered rats. J Cell Biochem 85:553–560.

Murata T, Yamaguchi M. 1998. Ca^{2+} administration stimulated the binding of AP-1 factor to the 5′-flanking region of the rat gene for the Ca^{2+}-binding protein regutcalcin. Biochem J 329:157–183.

Murata T, Yamaguchi M. 1999. Promoter characterization of the rat gene for Ca^{2+}-binding protein regucalcin. Transcriptional regulation by signaling factor. J Biol Chem 274:1277–1285.

Ng RCK, Peraino RA, Suki WN. 1982. Divalent cation transport in isolated tubules. Kidney Int 22:492–497.

Peleck SL, Charest DL, Mordret GP, Siow YL, Palaty C, Campbell D, Chaslton L, Samiei M, Sanghera JS. 1993. Networking with mitogen-activated protein kinases. Mol Cell Biochem 127(128):157–169.

Rasmussen J. 1970. Cell communication, calcium ion, and cyclic adenosine monophosphate. Science 170:404–412.

Rice EK, Tesck GH, Cao Z, Cooper ME, Metz CN, Bucala R, Atkins RC, Nikolic-Paterson DJ. 2003. Induction of MIF synthesis and secretion by tubular epithelial cells: A novel action of angiotensin? Kidney Int 63:1265–1275.

Shimokawa N, Yamaguchi M. 1993. Molecular cloning and sequencing of the cDNA codimg for calcium-binding protein regucalcin from rat liver. FEBS Lett 327:251–255.

Shimokawa N, Matsuda Y, Yamaguchi M. 1995. Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem 151:157–163.

Shimokawa N, Yamaguchi M. 1992. Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver. FEBS Lett 305:151–154.

Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita E. 1986. Staurosporine, a potent inhibitor of phospholipids/Ca^{2+}-dependent protein kinase. Biochem Biophys Res Commun 135:397–402.

Thiselton DL, McDowall J, Brandau O, Ramser J, d'Esposito F, Bhattacharya SS, Ross MT, Hardcastle AJ, Meindl A. 2002. An integrated, functionally annotated gene map of the DXS8026-ELK1 internal on human Xp11.3-Xp11.23: Potential hotspot for neurogenetic disorders. Genomics 79:560–572.

Tsurusaki Y, Yamaguchi M. 2003. Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells. J Cell Biochem 90: 619–626.

Tsurusaki Y, Yamaguchi M. 2004. Role of regucalcin in liver nuclear function: Binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression. Int J Mol Med 14:277–281.

Tsurusaki Y, Misawa H, Yamaguchi M. 2000. Translocation of regucalcin to rat liver nucleus: Involvement of nuclear protein kinase and phosphatase regulation. Int J Mol Med 6:655–660.

Van Heeswijk MPE, Geertsen JAM, Van Os CH. 1984. Kinetic properties of the ATP-dependent Ca^{2+} pump and the Na^{+}/Ca^{2+} exchange system in basolateral membranes from rat kidney cortex. J Membr Biol 79:19–31.

Van Os CH. 1987. Transcellular calcium transport in intestinal and renal epithelial cells. Biochim Biophys Acta 906:195–222.

Verheijen MHG, Defize LHK. 1997. Parathyroid hormone activates mitogen-activated protein kinase via a cAMP-mediated pathway independent of ras. J Biol Chem 272: 3423–3429.

Vincenzi FF. 1982. Pharmacology of calmodulin antagonism. In: Godfraind T, Albertini A, Paoletti R, editors. Calcium modulators. Amsterdam: Elsevier Biomedical Press. pp 67–80.

Wessendolf JHM, Ganfinkel S, Zhan X, Brown S, Maciag T. 1993. Identification of a nuclear localization sequence within the structure of the human interleukin-1α precursor. J Biol Chem 268:22100–22104.

Xue JH, Takahashi H, Yamaguchi M. 2000. Stimulatory effect of regucalcin on mitochondrial ATP-dependent calcium uptake activity in rat kidney cortex. J Cell Biochem 80:285–292.

Yamaguchi M. 1992. A novel Ca^{2+}-binding protein regucalcin and calcium inhibition. Regulatory role in liver cell function. In: Kohama K, editor. Calcium inhibition. Tokyo: Japan Sci Soc Press; Boca Raton: CRC Press. pp 19–41.

Yamaguchi M. 2000a. Role of regucalcin in calcium signaling. Life Sci 66:1763–1780.

Yamaguchi M. 2000b. The role of regucalcin in nuclear regulation of regenerating liver. Biochem Biophys Res Commun 276:1–6.

Yamaguchi M. 2005. Role of regucalcin in maintaining cell homeostasis and function (Review). Int J Mol Med (in press).

Yamaguchi M, Isogai M. 1993. Tissue concentration of calcium binding protein regucalcin in rats by enzyme linked immunoadsorbent assay. Mol Cell Biochem 122: 65–68.

Yamaguchi M, Kurota H. 1995. Expression of calcium-binding protein regucalcin mRNA in the kidney cortex of rats: The stimulation by calcium administration. Mol Cell Biochem 146:71–77.

Yamaguchi M, Yamamoto T. 1978. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull 26:1915–1918.