Conserved Mechanism of Negative Gene Regulation by Extracellular Calcium

Parathyroid Hormone Gene versus Atrial Natriuretic Polypeptide Gene

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Abstract

We found that a negative calcium-responsive element (nCaRE) originally reported in the human parathyroid hormone gene is conserved among several genes. The results of the present study show that expression of one of the genes, the rat atrial natriuretic polypeptide gene, was negatively regulated in the heart by extracellular calcium by using an in vivo infusion system. Moreover, transfection of the cultured cells revealed that this DNA element conferred negative regulation by extracellular calcium on the reporter gene. It is suggested that there is a gene family whose expression is negatively regulated by extracellular calcium through this conserved DNA motif, nCaRE. (*J. Clin. Invest.* 1992. 89:1268–1273.) Key words: negative calcium-responsive element • in vivo infusion system • transfection • gene family • nuclear protein

Introduction

Recently, the mechanisms of gene regulation have been extensively clarified. For example, steroid hormone receptors, AP-1 and cyclic AMP-response element binding protein have been identified (1–3). Almost all of the mechanisms stimulate gene transcription via formation of the specific DNA–protein complexes. On the other hand, the mechanisms of negative gene regulation or suppression of gene expression are not so well understood, with a few exceptions such as the glucocorticoid hormone receptor, which has been recently reported to suppress expression of some of its target genes through binding AP-1 (4).

We previously reported that expression of the parathyroid hormone (PTH)[1] gene is negatively regulated by extracellular calcium (5) as well as by 1,25-dihydroxyvitamin D_3 (6). We found that the 684-bp upstream region of the human PTH gene contains DNA elements that are necessary to mediate negative regulation by 1,25-dihydroxyvitamin D_3 (6). In this region, however, there is no DNA sequence homologous to the recently identified vitamin D–responsive element (7, 8) or the AP-1 binding site. On the other hand, we recently identified a DNA element that is required for negative regulation of the PTH gene expression by extracellular calcium (9).[2] This element, negative calcium-responsive element (nCaRE), which is located at the −3.5 kbp upstream region, consists of a 15-bp palindrome with thymidine clusters ahead of it (10).

Because we found that a nuclear protein that specifically binds the nCaRE in the human PTH gene (nCaRE–PTH) exists in a wide variety of cell types (9),[2] we assumed that there are other genes whose expressions are negatively regulated by extracellular calcium through the analogous sequence to the nCaRE. Then, we searched for the DNA sequence homologous to the nCaRE–PTH by using the EMBL gene bank. As a result, we found that five genes have DNA sequences that are identical or very homologous to the nCaRE–PTH in their 5′-flanking regions.

In the present study, moreover, we examined the effect of extracellular calcium on expression of one of these genes, the rat atrial natriuretic polypeptide (rANP) gene, in the heart. As expected, the present study showed that expression of the rANP gene is clearly negatively regulated by extracellular calcium. Transfection of the cultured cells with a reporter gene containing an rANP's DNA element that is homologous to the nCaRE (nCaRE–ANP) suggested that this DNA also functions as a negative calcium-responsive DNA element. Then, we propose here that there is a gene family whose expression is negatively regulated by extracellular calcium through binding of the nCaRE to a common putative nuclear protein(s).

Methods

Homology search by computer analysis. Using the core of the human parathyroid hormone (hPTH) gene's nCaRE, TGAGACAGGGTCTCA (9, 10), we searched for the DNA sequences that have ≥ 14 identical bases out of 15 bases of the nCaRE in the EMBL gene bank. In Fig. 1, only the genes that share such sequences in the 5′-flanking region are listed.

Animals and in vivo infusion experiments. Male Wistar rats (7–9 wk old) were used. The femoral vein was cannulated for continuous infusion for 48 h. The contents of infused solutions are as follows (5): calcium-free solution, 20 mM NaCl, 5 mM $MgCl_2$, 2.5 mM KCl, and 222 mM glucose; calcium-containing solution, the same composition as in calcium-free solution but containing 40 mM $CaCl_2$. The infusions were carried out at a speed of 2.4 ml/h (calcium load, 96 μmol/h). By using a similar condition, we previously showed that expression of the rat PTH gene was negatively regulated by extracellular calcium (5). 48 h after infusion, blood samples; heart, including both atrium and ventricle; and kidneys were obtained from an individual rat of each condition for the subsequent experiments. Plasma volume of the individual rat was calculated by the method described in reference 10. The

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1. *Abbreviations used in this paper:* BHK, baby hamster kidney; CAT, chloramphenicol acetyltransferase; (h)PTH, human parathyroid hormone; nCaRE, negative calcium-responsive element; (r)ANP, rat atrial natriuretic polypeptide; Tk, thymidine kinase.

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method for measuring heart rates or blood pressure is described in reference 11.

Analysis of RNA. Total RNA was extracted from the heart or the kidneys of the rat of each condition using guanidinium–cesium chloride method (12). In Northern blot analysis, 15 μg of total RNA of the heart or the kidneys from the individual rat was loaded on a 1.5% agarose, 2.2 M formaldehyde denaturing gel and then transferred onto a nylon membrane filter. Prehybridization and hybridization were performed at 42°C under the standard condition (6). The filter was washed at 65°C three times with 1× SSC for a total of 2 h. The nick-translated probe (5×10^8 cpm/μg) used in the experiments was derived from a 698-bp KpnI fragment of the rat renin cDNA (13) or a 368-bp HincII–StuI fragment of the rANP cDNA (14). The actin probe was described in reference 6.

Synthetic oligonucleotides and plasmid constructions. Oligonucleotides corresponding to the nCaRE in the hPTH gene and in the rANP gene were made by a DNA synthesizer (type 8770; Biosearch, San Rafael, CA). Synthetic complementary oligonucleotides were subsequently annealed and used for ligation to construct chimeric plasmids (1). Composition of each oligonucleotide is as follows.

Oligo nCaRE–PTH
(GATCC)TTTTTGAGACAGGGTCTCACTCTG(T)
(G)AAAAACTCTGTCCCAGAGTGAGAC(AGATC)
Oligo nCaRE–ANP
(GATCC)TTTTGGAGACAGGGTCTCATATAG(T)
(G)AAAACCTCTGTCCCAGAGTATATC(AGATC)
Oligo nCaRE–ANPmut
(GATCC)TTTTGATGCCAGGGCATCATATAG(T)
(G)AAAACTACGGTCCCGTAGTATATC(AGATC)

Bases in the parentheses are BamHI and XbaI cohesive ends to facilitate subsequent ligations to a BamHI–XbaI larger fragment of PUTKAT1 (10, 15). The mutated sequences in oligo nCaRE–ANPmut are underlined.

Transfection and chloramphenicol acetyltransferase (CAT) assay. Baby hamster kidney (BHK) cells were grown in (DME) supplemented with 10% FCS. 2 μg of each CAT plasmid was introduced into BHK cells by the DEAE–dextran method (16). After transfection, cells were equally split into three dishes to avoid difference in transfectional efficiency among dishes. 6 h later, cells were attached to the plate, and media were changed to DME with three different concentrations of extracellular calcium by adding EGTA. 40 h later, cells were harvested and the subsequent CAT assays were performed with 300 μg of cellular protein (9, 10). Average CAT activities were calculated and represented as mean±SEM after five separate transfections using ^{14}C scintillation counting (9). A CAT activity driven by PUTKAT1 (15) in the presence of 2.0 mM extracellular calcium is arbitrarily represented as 100.

Gel retardation assay. The methods for obtaining crude nuclear extracts (from BHK cells or from the rat heart) or for the gel retardation assay was described in reference 9. An oligonucleotide nCaRE–PTH or nCaRE–ANP was used as a probe after end-labeling by [γ-^{32}P]ATP and T4 polynucleotide kinase. 6 μg of nuclear protein as well as 2 μg of poly (dI)–(dC) were used in each assay. BHK nuclear extracts were prepared from the cells grown in 10% FCS-supplemented calcium-free DME (Gibco Laboratories, Grand Island, NY) in which $CaCl_2$ was added to maintain the cells with three different extracellular calcium concentrations (0.2, 1.5, and 3.0 mM) for 48 h and each of the extracts was incubated with the ^{32}P-end-labeled nCaRE–ANP (see Fig. 5, lanes *10–12*).

Results

Fig. 1 shows the genes that, in the 5′-flanking regions, have 14 identical DNA bases out of 15 bases of the palindromic DNA sequence of nCaRE[2] in the human PTH gene (TGAGACAGGGTCTCA). We have found that three genes have identical DNA sequences and two genes have very homologous (14/15) DNA ones in their 5′-flanking regions. Very interestingly, not only the palindromic portion but also thymidine clusters or thymidine-rich sequences ahead of it are very well conserved among them, suggesting that these thymidines also play an important regulatory role.[2] So far, expression of these genes has not been reported to be negatively regulated by extracellular calcium as is seen in the PTH gene. However, some investigators demonstrated that secretion of rANP was suppressed by extracellular calcium (17), though the effect of extracellular calcium on its transcription as well as on its secretion is still controversial (17–19).

To examine the possibility that expression of the rANP gene is suppressed by extracellular calcium in heart, we used the in vivo rat infusion system (5). Using this system (5), we previously showed that expression of the rat PTH gene is negatively regulated by extracellular calcium in the parathyroid gland. By Northern blot analyses, we compared the level of ANP mRNA obtained from the rat heart infused with calcium-free solution to that obtained from the rat heart infused with calcium-containing solution. These solutions were infused for 48 h. During this period, plasma volume of the Ca-free rat (4.5±0.4 ml/100 g body wt) was not significantly different from that of the Ca-plus rat (4.6±0.1 ml/100 g body wt) (10). Mean blood pressure or heart rate in conscious rats measured by the method described in reference 11 did not significantly differ between these two groups either (data not shown). These observations potentiate the validity of our analyses, because we can attribute the difference in the levels of rANP mRNA, if any, to the difference in the level of extracellular calcium (see below). As shown in Fig. 2, the level of rANP mRNA from the heart of the calcium-infused rats (serum calcium level was 12.5–12.6 mg/dl) was markedly lower compared with that from the rats without calcium (serum calcium level was 9.6–9.8 mg/dl). On the other hand, the level of actin mRNA did not significantly differ between the two with and without calcium.

Because secretion of renin is also known to be suppressed by extracellular calcium (20), we also studied the effect of calcium on the level of renin mRNA in the rat kidney. As shown in Fig. 3, the increase in extracellular calcium did not affect the level of renin mRNA in the rat kidney. In a computer search, we found that the rat renin gene does not have DNA sequences > 80% homologous to the nCaRE, including thymidine clusters (data not shown).

We next synthesized a short DNA sequence corresponding to the putative nCaRE in the rANP gene (nCaRE–ANP) as well as a DNA sequence where five bases are mutated (nCaRE–ANPmut). The nCaRE–ANP has one base different from the palindromic portion of the hPTH gene's nCaRE (T to G, see Fig. 1) and similar thymidine clusters ahead of it. Each of these DNA sequences was ligated to the upstream portion of the herpes viral thymidine kinase (Tk) gene promoter-CAT plasmid (15)[2] and introduced into BHK cells. As shown in Fig. 4, reduction of extracellular calcium concentrations from 2.0 to 1.0 mM significantly elevated CAT activity driven by the plasmid containing nCaRE-ANP as much as threefold to ~ 30% of the CAT activity elicited by the parental PUTKAT1. Similar results were obtained using the hPTH gene's nCaRE (nCaRE–PTH) Tk promoter-CAT plasmid.[2] On the other hand, CAT activities driven by the parental PUTKAT1 that has mutated or by no nCaRE–ANP were not affected by the same procedure. Although we have used a rather higher amount of the cellular protein (300 μg) to clearly show the difference in CAT

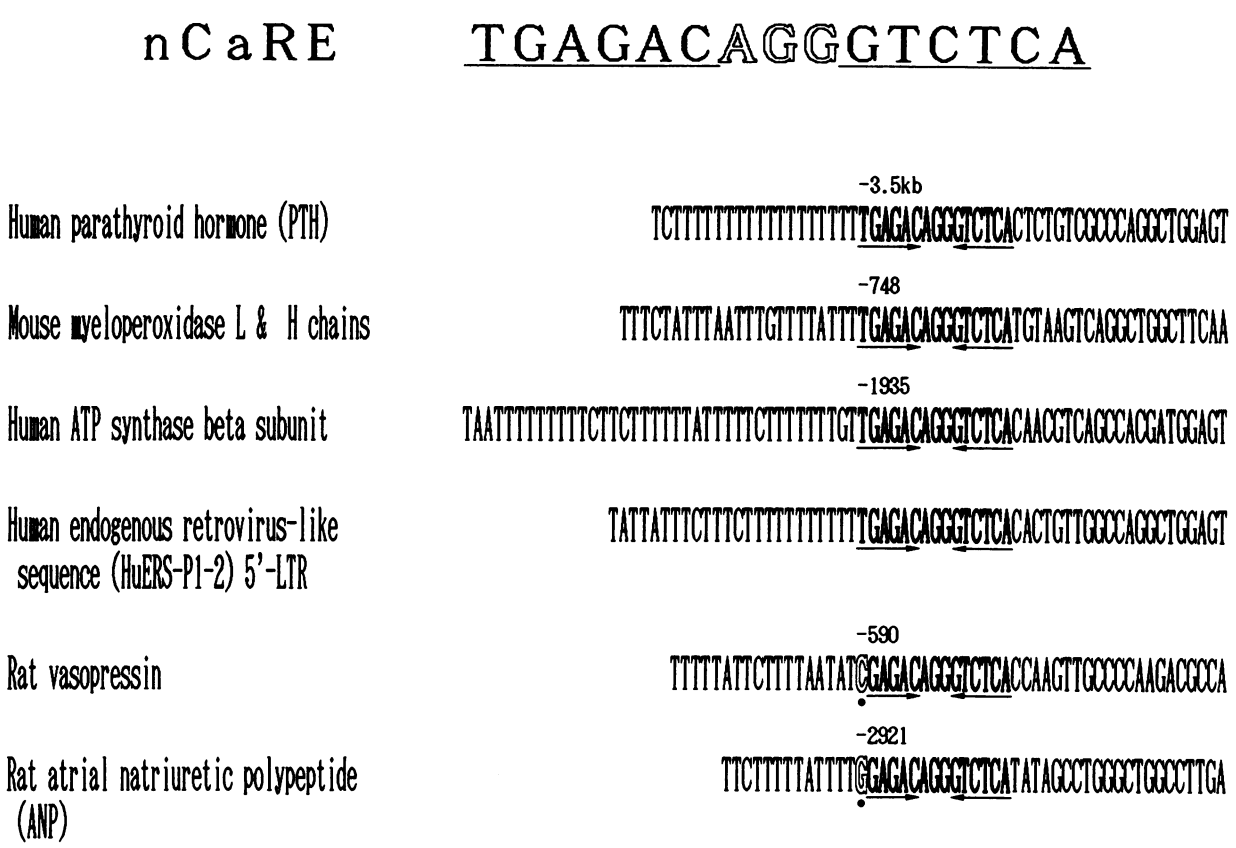

Figure 1. Computer search for the genes that contain DNA sequences homologous to the nCaRE of the hPTH gene in their 5'-flanking regions. On the top, a DNA sequence of the nCaRE in the hPTH gene (9)[2] is shown. The upper four genes contain an identical 15-bp palindromic sequence indicated by arrows. The lower two genes share 14 identical bases within the 15-bp palindrome. In the rat vasopressin gene, the first thymidine is converted to cytosine, and in the rANP gene, it is converted to guanine.

activities created by these plasmids, a smaller amount of the protein (100 μg) obtained from the cells transfected with the parental PUTKAT1 (in this condition, conversion rate from the substrate chloramphenicol to its acetylated form was ~ 10% compared with that of 20% shown in Fig. 4) created CAT activities unaffected by extracellular calcium (data not shown).

These results suggest that elevated extracellular calcium, but not secondary changes of the circulating level of PTH or calcitonin, specifically suppressed the level of ANP mRNA in the heart, probably at least in part through the nCaRE–ANP.

To show that a nuclear protein in the rat heart cell binds the nCaRE–ANP, gel retardation assays were performed. As shown in Fig. 5, the interaction between a rat heart nuclear protein and the nCaRE–ANP created two bands that were competed out by a 80-fold molar excess of this oligonucleotide (lanes *2* and *3*). The sequence-specificity of this binding was further confirmed when we used a BHK nuclear protein in the assay; as shown in lanes *4–9*, not only the nCaRE–ANP but also nCaRE–PTH competed the binding of the nCaRE–ANP, whereas the nCaRE–ANPmut did not. Compared with the upper band, the lower band was less efficiently competed by the nCaREs (lanes *4–8*). This might be due to the smaller amount of the competitors used (20-fold in lanes *6* and *8* vs. 80-fold in lane *3*) or might reflect different amounts of the nuclear protein specifically binding to the nCaRE–ANP between BHK and the rat heart cells (see Discussion). Moreover, a combination of a BHK nuclear protein and the nCaRE–PTH, as well as that of a rat heart nuclear protein and the nCaRE–ANP, generated these two protein–DNA complexes at similar migration positions, strongly suggesting that the nuclear proteins involved are identical. Further, when a nuclear protein was obtained from BHK cells grown in conditions with three different extracellular calcium concentrations, the nuclear protein from the higher extracellular calcium condition exhibited a stronger binding to the nCaRE–ANP (Fig. 5, lanes *10–12*). Taken together, negative gene regulation of the rANP gene by extracellular calcium may well be mediated, at least in part, through an increased and sequence-specific binding of the nCaRE–ANP to a nuclear protein in the presence of higher extracellular calcium concentration, as is the case with the regulation of the hPTH gene.[2]

Discussion

In this report, we have shown that an nCaRE originally found in the hPTH gene[2] is actually distributed among the upstream regions of several genes. Expression of one of these genes, the rANP gene, was shown to be negatively regulated by extracellular calcium as is seen in the PTH gene. The nCaRE consists of a 15-bp palindrome with three asymmetrical bases, AGG, in the middle of it. In a previous report,[2] we showed that this 15-bp palindrome plus thymidine-rich sequences are important for the negative regulation of expression of the hPTH gene by extracellular calcium. This contention is supported by our find-

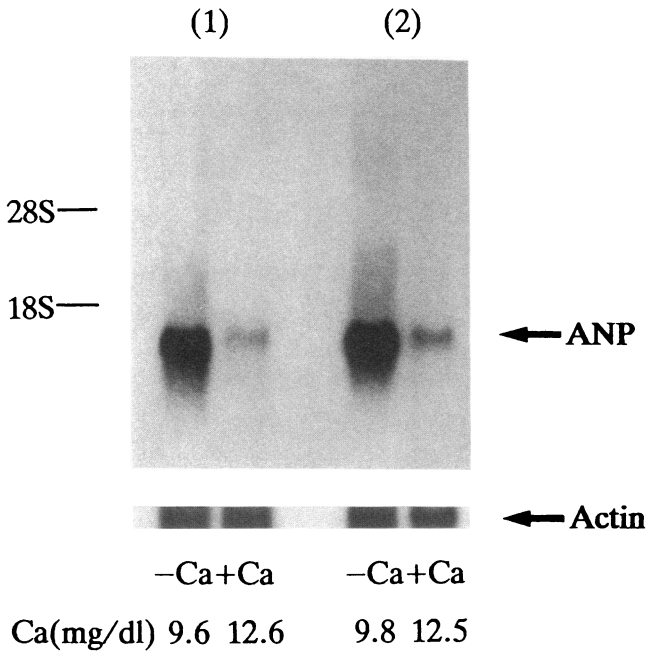

Figure 2. Negative regulation of the rANP gene by extracellular calcium. The results of two separate experiments are shown. 15 μg of total RNA obtained from the heart of the individual rat infused with calcium-free solution [*left lanes* of (*1*) and (*2*)] and with calcium-containing solution (*right lanes*) are compared by Northern blot analysis. The levels of ANP and actin mRNA are separately shown. At the bottom, serum total calcium concentrations of the rats of each condition are indicated.

ing in Fig. 1 that not only the palindromic portion but also the thymidine clusters ahead of it are very well conserved among these genes. The important role of the palindrome as well as the flanking sequences in gene regulation were previously shown in the case of binding of AP-1 or cyclic AMP-response element binding protein (2, 21). Indeed, gel retardation assay (Fig. 5) reveals that both the nCaRE–ANP and nCaRE–PTH yield two specific protein–DNA complexes, which might imply monomer and dimer/multimer formation. When we use a smaller amount of the nuclear protein we are able to observe only the lower band (data not shown; see reference 9). Therefore, the upper band (Fig. 5), which presumably reflects a dimer/multimer complex generated subsequently after a monomer is formed, may be competed more easily by the nCaRE–ANP. This hypothesis may explain why the lower band in Fig. 5 (especially in lanes *6* and *8*) is less efficiently competed by the nCaRE–ANP compared with the upper band. Such interpretation is based on the observation that the nCaREs share a palindromic structure that is well known to facilitate dimer/multimer protein–DNA complex formation, but the detailed mechanism of the binding remains to be elucidated. On the other hand, the T clusters or T-rich sequences observed in the nCaRE have not been recognized in any *cis*-acting DNA elements so far. Presumably, these T clusters are important either to loop out the intervening DNA sequences for an nCaRE-binding protein(s) to directly act on the basic transcription apparatus such as a TATA box-binding factor (22) or to facilitate access of transcriptional apparatus to the palindromic portion to which the nuclear protein(s) actually binds. Moreover, conservation of the center sequence, AGG, among these genes suggests that these three bases are also important for its function.

We previously showed that negative regulation of gene expression by extracellular calcium is caused by the sequence-specific and increased binding of the nCaRE(–PTH) to a nuclear protein(s) using gel retardation assays,[2] which was similarly reproduced in this paper. Although the minimal sequence requirement for such DNA–protein binding is not identified, a mutation of the first thymidine to guanine does not affect such binding (Fig. 5). Expression of other genes shown in Fig. 1 might also be regulated by extracellular calcium, although the physiological significance is unclear so far, even in the case of the ANP gene. We have recently obtained data showing that rat vasopressin gene expression in the brain is also suppressed by extracellular calcium by using similar experimental strategy (manuscript in preparation). One plausible speculation for the nCaRE's role especially in the ANP or vasopressin gene is that the intracellular signal transduction system functioning at the upstream of nCaRE-binding protein in the cells expressing these genes might be activated not only by extracellular calcium but also by other physiological extracellular stimuli such as tension or osmolality. Actually, there have been many reports describing a close relationship between these stimuli and extracellular calcium (23, 24).

Suppression of the rANP gene by extracellular calcium is mediated at least in part by the nCaRE–ANP because the binding pattern of the oligonucleotides to the nuclear protein correlates quite well to the pattern of extracellular calcium–mediated gene regulation conferred by such oligonucleotides. In other words, CAT activities driven only by the plasmid containing the oligonucleotides that specifically bind to a nuclear

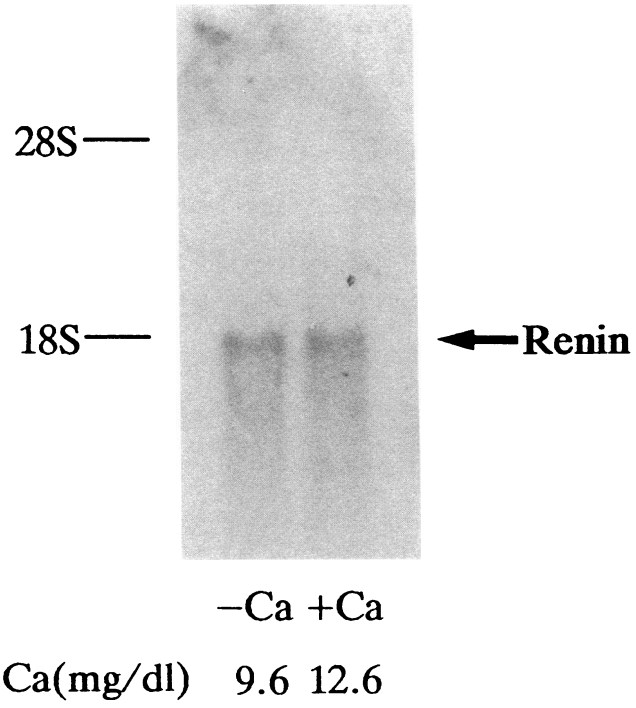

Figure 3. The level of the rat renin mRNA in kidneys. 15 μg of total RNA from the kidneys of the individual rat was analyzed. The experimental condition was the same as the one shown in the left part of Fig. 2.

protein were increased when extracellular calcium concentration was decreased. However, it did not reach the level elicited by a parental plasmid where no nCaRE was included (PUTKAT1). Such incomplete recovery of the CAT activity was also recognized when we used the nCaRE–PTH–PUTKAT1 previously.[2] Even when we used 0.2 mM extracellular calcium adjusted by adding $CaCl_2$ to the Ca-free DME, CAT activity driven by these plasmids remained at the same level as that in the presence of 1.0 mM extracellular calcium (data not shown). The reasons for these observations might be twofold. First, in our plasmid, the distance between the nCaRE and the promoter region necessary for basal transcription might be too close to elicit physiological extracellular calcium–mediated transcriptional regulation. The nCaRE–PTH and the nCaRE–ANP normally exist in a far upstream region from their own transcriptional start site, respectively (Fig. 1). The plasmid in our previous report[2] containing the nCaRE–PTH linked to the hPTH promoter- (but not the Tk promoter-) CAT gene produced the CAT activity almost equal to that generated by the parental PTH–CAT plasmid containing no nCaRE–PTH in 1.0 mM extracellular calcium concentration. In that case, the distance between nCaRE and the hPTH promoter is ~ 650 bp, in contrast to a distance of ~ 50 bp between nCaRE–ANP and Tk promoter in our present construct. Presumably, a smaller distance between the nCaRE and the promoter region would yield stronger suppression of the CAT activity by extracellular calcium. Second, the basic transcriptional apparatus functioning at the Tk promoter region might interact more tightly with the nCaRE-binding protein than the apparatus functioning at the PTH promoter region, leading to weaker reactivation of the CAT activity even in the presence of lower extracellular calcium. Both possibilities remain to be elucidated. Further, we cannot rule out the possibility that not only the nCaRE–ANP but also another DNA element(s) is required for full suppression of the rANP gene expression by extracellular calcium. This issue will be clarified more extensively by using the rANP promoter instead of the Tk promoter, which is currently under investigation in our laboratory.

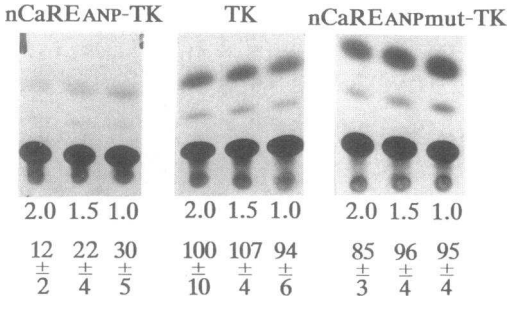

Figure 4. CAT assays in BHK cells transfected with the reporter genes containing the nCaRE-ANP, nCaRE-ANPmut, or no nCaRE. After transfection of the cells by the DEAE–dextran method, cells were split into three dishes to avoid differences in transfectional efficiency among the dishes. The concentrations of extracellular calcium were manipulated by adding three different concentrations of EGTA to DME containing 10% FCS.[2] The concentrations of extracellular calcium in each condition are shown. Average CAT activities after five independent transfections are shown in each condition as mean±SEM. The mean CAT activity driven by the parental PUTKAT1 is arbitrarily represented as 100. In this figure, one of the typical results is shown.

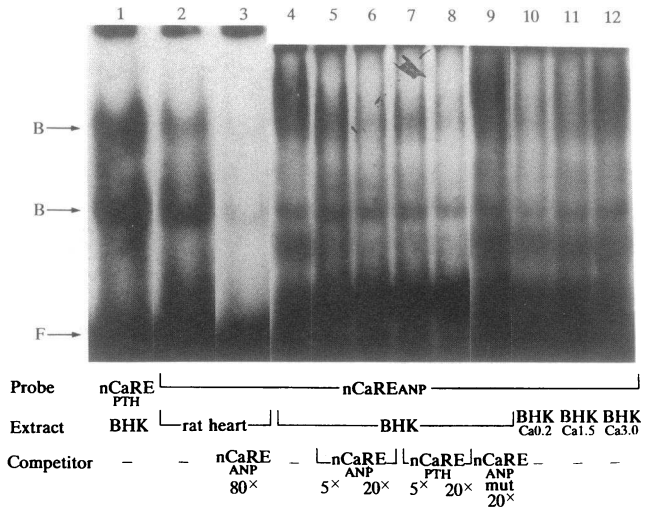

Figure 5. Gel retardation assays using nuclear extract from BHK cells and the rat heart. The nCaRE–PTH (lane *1*) or the nCaRE–ANP (lanes *2–12*) was used as a probe. A 5-fold (lanes *5* and *7*), 20-fold (lanes *6* and *8*), and 80-fold (lane *3*) molar excess of the nonradiolabeled nCaRE–ANP was added as a competitor. In lanes *10–12,* BHK nuclear extracts were obtained from the cells grown in DME containing 0.2 mM (lane *10*), 1.5 mM (lane *11*), and 3.0 mM (lane *12*) extracellular calcium. In lanes *1* and *4–10,* extracellular calcium concentration was 2.0 mM.

We have not examined the possibility that the homologous sequence to the nCaRE is seen in the PTH or ANP gene of other species. Although DNA sequences of many genes have been completely identified, including their upstream portions, such identification may not cover the far upstream region where the nCaRE may usually be located (Fig. 1).

The membrane machineries that sense the level of extracellular calcium or intracellular signals followed by changes of extracellular calcium leading to transcriptional suppression by extracellular calcium have not been identified in this study. In many cells, including atrial and ventricular, changes in the level of extracellular calcium are never supposed to alter the level of intracellular calcium, which is in good agreement with the observations that the level of intracellular calcium is by far less abundant compared with the level of extracellular calcium (25). In this regard, parathyroid cells are one of few exceptions, in that changes of the level of extracellular calcium easily alter the level of intracellular calcium (26). This might suggest that the intracellular signal for extracellular calcium–mediated transcriptional suppression is intracellular calcium, because the first example of this kind of regulation was seen in the PTH gene, which is exclusively expressed in the parathyroid cells. However, the results of this study contradict this notion because negative regulation of the rANP gene was seen in the heart cells. We previously showed that an nCaRE-binding protein(s) is actually distributed among various types of cells (9, 10). As shown in Fig. 5, the rat heart cells also possess this protein, which binds to the nCaRE–ANP in a sequence-specific and extracellular calcium–dependent manner as the nCaRE–PTH–binding nuclear protein does (9).[2] Presumably, the signal transduction machinery activated by changes of extracellular calcium is also widely distributed among many cell types.

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