TÜBİTAK

Research Article

Turk. J. Vet. Anim. Sci.
2010; 34(3): 249-254
© TÜBİTAK
doi:10.3906/vet-0809-27

Expression and tissue distribution of extracellular calcium-sensing receptor (CaSR) mRNA in chickens

Guangcun DENG[1,2], Jianmin BI[1], Jian QIAO[1,*], Guiei HE[1], Boning WU[1], Miaojie ZHANG[1], Nana LV[1]

[1]College of Veterinary Medicine, China Agricultural University Beijing 100193 - CHINA

[2]College of Life Science, Ningxia University Yinchuan, 750021 - CHINA

Received: 18.09.2008

Abstract: Extracellular calcium-sensing receptor (CaSR) is a recently discovered G-protein-coupled receptor that may play a key role in regulating parathyroid hormone secretion and calcium homeostasis. In the present study, based on the reported sequence of the chicken CaSR gene (GenBank accession: XM_416491), specific primers were designed and synthesized, and CaSR gene mRNA expression and tissue distribution in chickens were analyzed using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. It was observed that CaSR mRNA was expressed in the kidney and parathyroid to a greater degree than in the small intestine, oviduct, and brain ($P < 0.01$). Sequence analysis of the PCR products obtained from the 5 tissues showed that these cDNAs shared 100% homology with each other and the corresponding region of the reported CaSR cDNA. The results may provide a basis and additional insight for understanding the physiological function of CaSR in chickens.

Key words: Extracellular calcium-sensing receptor (CaSR), parathyroid hormone, tissue distribution, calcium homeostasis

Introduction

It is well known that intracellular calcium ions are very important for a large range of cellular processes, including muscle contraction, hormonal secretion, and cell division, because they act as vital intracellular second messengers (1,2). In contrast to intracellular calcium, extracellular calcium is maintained at a nearly constant physiological concentration and is rigidly controlled by homeostatic mechanisms in the healthy body (3). In 1993 Brown et al. (4) first reported an extracellular calcium-sensing receptor (CaSR) in the bovine parathyroid gland and proved that it belongs to the G-protein-coupled receptor superfamily that includes 8 metabotropic glutamate receptors (mGluR1-8) and 2 γ-aminobutyric acid receptor subunits (GABAB1 and GABAB2). Moreover, to date, all cloned CaSRs have had a common overall topology that includes a very large (~600 amino acids) NH_2-terminal extracellular domain (ECD) and a central core of some 250 amino acids with 7 predicted transmembrane domains (TMDs) (4-8). The molecular identification of the extracellular calcium-sensing receptor has opened up the possibility that Ca^{2+} might also function as a

* E-mail: qiaojian010@yahoo.cn

messenger outside the cell. (2,4). Through the CaSR the parathyroid gland maintains serum calcium concentrations within a very narrow physiological range by modulating the minute-to-minute release of parathyroid hormone (PTH) into the circulation (7).

Since Brown et al. (4) first cloned and characterized the CaSR from the bovine parathyroid gland in 1993, CaSRs from diverse species, including mammals, birds, amphibians, and reptiles, have been reported and characterized (6,9-15). It has also been shown that the CaSR is expressed in multiple tissues, including such calcium homeostasis tissues as the parathyroid gland, kidney, intestine, and bone, as well as in non-calcium-homeostasis tissues, such as the brain, pancreas, lungs, stomach, and cardiovascular system (14,16-20). Recent research focused on the role of the CaSR as a regulator of parathyroid hormone synthesis and secretion, as well as systemic calcium homeostasis; however, to date, the physiological function of the CaSR in non-calcium-homeostasis tissues has remained obscure.

It is thought that the chicken may be a useful model for studying calcium homeostasis because of the extremely rapid initial growth of the skeleton and massive calcium turnover during eggshell formation, in which the CaSR may play a key role (21). Clarification of the mechanisms underlying these biological processes requires detailed study of CaSR expression and tissue distribution in chickens. The present study aimed to analyze CaSR expression and tissue distribution in chickens based on semiquantitative reverse-transcription PCR. The results might contribute to the elucidation of the CaSR's biological function in systemic calcium homeostasis and non-calcium-homeostasis tissues.

Materials and methods

Animals

The study included 5 high-yield laying chickens obtained from a local farm. The chickens were sacrificed according to the humane practices of animal care established by the China Agricultural University Standing Committee on Animals. Tissues, including the heart, liver, spleen, lungs, kidneys, small intestine, pancreas, stomach, parathyroid gland, oviduct, and brain, were harvested and immediately stored at –70 °C until use.

RNA extraction and reverse transcription

To determine if CaSR mRNA is expressed in different tissues total RNA in the tissues was isolated with TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. The quality and quantity of total RNA obtained were estimated by agarose gel electrophoresis and UV spectrophotometry. The OD260:OD280 ratio of total RNA from different tissues was 1:81, 1:85, 1:82, 1:91, 1:90, 1:87, 1:84, 1:93, 1:89, 1:81, and 1:86 for the heart, liver, spleen, lungs, kidneys, small intestine, pancreas, stomach, parathyroid gland, oviduct and brain, respectively. To prevent genomic DNA contamination of the RNA preparation, 2 U RNAse-free DNase (TaKaRa) was added to each RNA sample and incubated at 37 °C for 30 min. Next, 10 μL of DNase inactivation reagent was added and incubated for 2 min at room temperature to inactivate the DNase. Subsequently, total RNA (10 μL) was used for single-strand cDNA synthesis in a reaction volume of 25 μL, using 2 μL of 50 pmol oligo $(dT)_{15}$ primers (TaKaRa). After denaturation at 70 °C for 5 min, 5 μL of M-MLV RT 5 × reaction buffer, 1 μL (200 U) of M-MLV reverse transcriptase (Promega), 1 μL of ribonuclease inhibitor, 4 μL of 10 mM dNTPs (TaKaRa), and 2 μL of nuclease-free water were sequentially added, and incubated at 42 °C for 1 h. The reverse transcriptase was inactivated by heating at 95 °C for 5 min.

PCR analysis

Based on the reported sequence of the chicken CaSR gene (GenBank accession: XM_416491) and β-actin (GenBank accession: L08165), specific primers were designed and synthesized for CaSR and β-actin using Primer v.5.0 software (Table 1). In order to compare the level of CaSR mRNA expression in the different tissues studied β-actin was used as an internal control under the same RT-PCR conditions.

Table 1. Sequence of primers used in RT-PCR.

Primer	Sequence	Accession number
CaSR F	ACGCTTTCAAGGTGGC	XM_416491
CaSR R	GGGCTGGATGCTGTCTG	XM_416491
β-actin F	ACACGGTATTGTCACCAACT	L08165
β-actin R	TAACACCATCACCAGAGTCC	L08165

The expected fragments were 400 bp and 263 bp, respectively. The resultant first-strand cDNA was then used as a template for the PCR procedure. PCR was carried out in a total volume of 25 μL of a buffer solution containing the following: 5 μL of cDNA (template), 2.5 μL of 10 × PCR buffer, 0.5 μL of forward primer, 0.5 μL of reverse primer, 3 μL of 2.5 mM dNTPs, 0.5 μL of Taq polymerase (TaKaRa), and 13 μL of ddH_2O. The final mixture for both CaSR and β-actin were run for 30 PCR cycles (94 °C for 40 s, 58 °C for 40 s, and 72 °C for 45 s), followed by a 10-min final extension at 72 °C. As a negative control, the cDNA template was replaced with an equivalent amount of water. Expected fragments were purified with a DNA fragment purification kit and ligated into the pMD18-T vector via a T-A cloning strategy (TaKaRa). Competent cells were then transformed and plated on ampicillin-containing agar. Transformed cells were identified after overnight growth at 37 °C using colony PCR.

The presence of a 400-bp fragment was indicative of a positive clone. Plasmid from positive colonies was extracted and used as a template for further PCR identification and sequencing. PCR products were electrophoresed in 2% agarose gel and target bands were analyzed using image analysis software (QUANTITY ONE, Bio-Rad). The abundance ratio of the target gene and β-actin was considered to be the relative quantity of the gene. Statistical significance of differences in CaSR mRNA between different tissues was analyzed by Duncan's multiple comparisons. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SD. SAS v.8.02 (SAS Institute, Cary, NC, USA) was used to perform all calculations.

Results

RT-PCR analysis of CaSR mRNA expression in different tissues

In order to determine if CaSR was expressed in different tissues we used a pair of specific primers, as mentioned above, based on the reported chicken CaSR gene sequence. The results showed that the expected fragment was not identified in the heart, liver, spleen, lungs, pancreas, stomach, or negative control. However, as shown in Figure 1, reverse transcription and PCR amplification of total RNA with CaSR-specific primers in the parathyroid gland, kidneys, small intestine, oviduct, and brain yielded the expected 400-bp product corresponding to the CaSR gene, and the RT-PCR product was detected in 2% agarose gel. These results indicate that CaSR was expressed to a greater degree in the parathyroid gland, kidneys, and small intestine than in the oviduct and brain (Table 2 and Figure 2) ($P < 0.05$), suggesting a different level of expression in these 5 tissues.

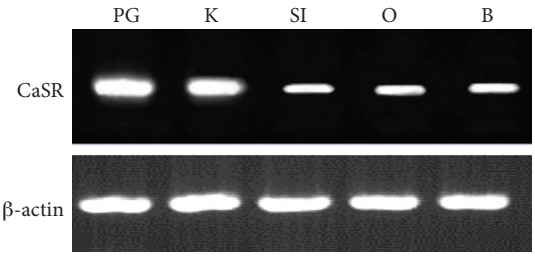

Figure 1. Tissue expression of CaSR mRNA. PG- Parathyroid gland, K- Kidney, SI- Small intestine, O- Ooviduct, B- Brain.

Table 2. Expression of calcium-sensing receptor mRNA in different tissues in chicken (n = 3).

	Tissue				
	Parathyroid gland	Kidney	Small intestine	Oviduct	Brain
Expression levels	0.52 ± 0.03^a	0.51 ± 0.03^a	0.32 ± 0.02^b	0.28 ± 0.02^c	0.27 ± 0.03^c

Note. The numbers in the table indicate: mean ± SD (sample size). The same letters following values indicate that the differences were not significant ($P > 0.05$). Different letters indicate that the differences were statistically significant ($P < 0.05$). Statistical significance of differences of CaSR mRNA between different tissues was analyzed by Duncan's multiple comparisons.

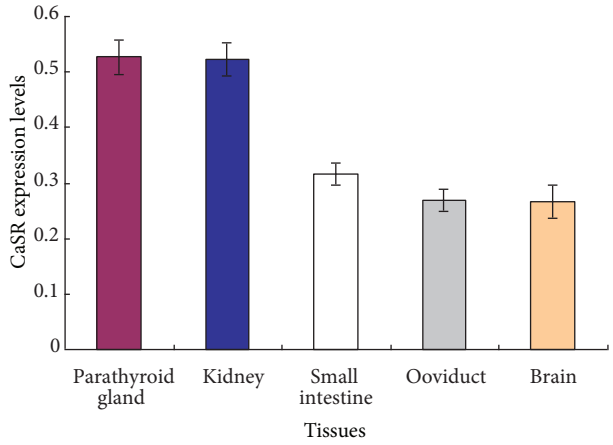

Figure 2. Expression of calcium-sensing receptor mRNA in different tissues in chicken.

Nucleotide sequencing of the RT-PCR product

Sequence analysis (Figure 3) of the 400-bp PCR products obtained from the parathyroid gland, kidneys, small intestine, oviduct, and brain revealed that these cDNAs shared 100% homology with each other and the corresponding region of the reported CaSR cDNA.

Discussion

In the present study we demonstrated the presence and tissue distribution of the extracellular calcium-sensing receptor in chickens based on a semiquantitative RT-PCR. The present results

```
xm_416491.seq   ACGCTTTCAAGGTGGCCGCCAGGGCCACGCTGAGACGCAGCAATGTGTCACGCAAGCGTT 3000
K-CaSR.seq      ------------------------------------------------------------
SI-CaSR.seq     ------------------------------------------------------------
P-CaSR.seq      ------------------------------------------------------------
B-CaSR.seq      ------------------------------------------------------------

xm_416491.seq   CCAACAGCCTCGGAGGTTCCACCGGTTCCACCCCATCCTCCTCCATCAGCAGCAAGAGCA 3060
K-CaSR.seq      ------------------------------------------------------------
SI-CaSR.seq     ------------------------------------------------------------
P-CaSR.seq      ------------------------------------------------------------
B-CaSR.seq      ------------------------------------------------------------

xm_416491.seq   ACCATGAAGACCCTTTTCCTCTACCGGCTTCTGCTGAGCGGCAGCGGCAGCAGCAGCGTG 3120
K-CaSR.seq      ------------------------------------------------------------
SI-CaSR.seq     ------------------------------------------------------------
P-CaSR.seq      ------------------------------------------------------------
B-CaSR.seq      ------------------------------------------------------------

xm_416491.seq   GGTGCAAGCAGAAGGTCAGCTTTGGGAGTGGTACGGTCACCTTGTCACTGAGTTTTGAGG 3180
K-CaSR.seq      ------------------------------------------------------------
SI-CaSR.seq     ------------------------------------------------------------
P-CaSR.seq      ------------------------------------------------------------
B-CaSR.seq      ------------------------------------------------------------

xm_416491.seq   AGCCACAGAAGAACGCCATGGCCAACAGGAACGCCAAGCGCAGGAACTCCCTGGAGGCCC 3240
K-CaSR.seq      ------------------------------------------------------------
SI-CaSR.seq     ------------------------------------------------------------
P-CaSR.seq      ------------------------------------------------------------
B-CaSR.seq      ------------------------------------------------------------

xm_416491.seq   AGAACAGCGATGACAGCCTGATGCGGCACAGGGCCCTGCTCGCTCTACAGAACAGCGAGT 3300
K-CaSR.seq      ------------------------------------------------------------
SI-CaSR.seq     ------------------------------------------------------------
P-CaSR.seq      ------------------------------------------------------------
B-CaSR.seq      ------------------------------------------------------------

xm_416491.seq   CCCTCAGTGCCGAGCCTGGCTTCCAGACAGCATCCAGCCC                  3339
K-CaSR.seq      ----------------------------------------
SI-CaSR.seq     ----------------------------------------
P-CaSR.seq      ----------------------------------------
B-CaSR.seq      ----------------------------------------
```

Figure 3. Nucleotide sequences of the CaSR RT-PCR products amplified from parathyroid gland, kidney, small intestine, oviduct, and brain. Forward and reverse primer sequences are shown in the boxes.

corroborate those of Diaz et al. (11). It has also been reported that the chicken parathyroid CaSR shares a high degree of homology (84% amino acid identity) with the human CaSR and exhibits a similar topology (11). Our data show that the specific fragment of CaSR from the parathyroid gland, kidneys, small intestine, oviduct, and brain share 100% homology with each other and the corresponding region of the reported CaSR cDNA. This shows that CaSR is rather conservative in different tissues as well as in different species, as it has a similar biological function.

In mammals the parathyroid gland, intestine, bones, and kidneys play a pivotal role in systematic calcium homeostasis by means of the CaSR (22-24). Most recent studies have focused on the molecular mechanisms of the CaSR in regulating PTH secretion, renal function, and maintenance of a stable serum calcium concentration (24-28). To date, the physiological function of CaSR in calcium homeostasis tissues has been well understood; however, recent research shows that CaSR is present in many non-calcium-homeostasis tissues, such as the brain, pancreas, lungs, stomach, skin, prostate, and cardiovascular system (14,16,17,19,20,25,29). Nevertheless, the biological function poorly understood. A growing body of evidence suggests that most tissues that do not participate in systemic calcium homeostasis might participate in the regulation of "local" calcium homeostasis within their immediate microenvironments via cell surface CaSR, and that they adjust the translocation of either ion so as to adjust the local ionic composition in a physiologically relevant manner (6).

In the present study we identified the presence of CaSR mRNA in the brain and oviduct (as non-calcium-homeostasis tissues), in addition to the kidneys, small intestine, and parathyroid gland; expression of CaSR mRNA in the brain suggests that CaSR may be associated with neuronal activity by acting as a local signal for cells within specific microenvironments (30). To the best of our knowledge the avian eggshell is formed during the passage of the egg through the oviduct. Most calcium deposition in the eggshell occurs during this time (21); the presence of CaSR mRNA in the oviduct indicates that CaSR probably plays a key role in this process, although the molecular mechanism underlying this remains to be elucidated.

In conclusion, extremely rapid initial growth of the skeleton, and massive spatial and temporal calcium turnover during eggshell formation makes the chicken a useful model for studying calcium homeostasis (21); however, CaSR gene expression and tissue distribution, the relationship between CaSR gene expression and PTH secretion, and calcium homeostasis have not been fully elucidated. Thus, the present results should contribute to our understanding of these mechanisms.

Acknowledgements

We thank the National Natural Science Foundation of China for its financial support (grant no. 30771621).

References

1. Brown, E.M.: Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. Physiol. Rev., 1991; 71: 371-411.
2. Zhao, X., Pei, Z.M., He, Y.K.: Extracellular Ca^{2+} signaling: first messenger in animals and plants. Yi Chuan, 2007; 29: 269-275.
3. Spurr, N.K.: Genetics of calcium-sensing - regulation of calcium levels in the body. Curr. Opin. Pharmacol., 2003; 3: 291-294.
4. Brown, E.M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M.A., Lytton J., Hebert, S.C.: Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. Nature, 1993; 366: 575-580.
5. Chattopadhyay, N.: Biochemistry, physiology and pathophysiology of the extracellular calcium-sensing receptor. Int. J. Biochem. Cell Biol., 2000; 32: 789-804.
6. Brown, E.M., MacLeod, R.J.: Extracellular calcium sensing and extracellular calcium signaling. Physiol. Rev., 2001; 81: 239-297.
7. Chen, R.A., Goodman, W.G.: Role of the calcium-sensing receptor in parathyroid gland physiology. Am. J. Physiol. Renal Physiol., 2004; 286: 1005-1011.
8. Ray, K., Adipietro, K.A., Chen, C., Northup, J.K.: Elucidation of the role of peptide linker in calcium-sensing receptor activation process. J. Biol. Chem., 2007; 282: 5310-5317.

9. Garrett, J.E., Capuano, I.V., Hammerland, L.G., Hung, B.C.P., Brown, E.M., Hebert, S.C., Nemeth, E.F., Fuller, F.: Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. J. Biol. Chem., 1995; 270: 12919-12925.

10. Riccardi, D., Park, J., Lee, W.S., Gamba, G., Brown, E.M., Hebert, S.C.: Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. Proc. Natl. Acad. Sci, 1995; 92: 131-135.

11. Diaz, R., Hurwitz, S., Chattopadhyay, N., Pines, M., Yang, Y., Kifor, O., Einat, M.S., Butters, R., Hebert S.C., Brown, E.M.: Cloning, expression, and tissue localization of the calcium-sensing receptor in chicken (*Gallus domesticus*). Am. J. Physiol., 1997; 273: 1008-1016.

12. van den Hurk, M.J., Ouwens, D.T., Scheenen, W.J., Limburg, V., Gellekink, H., Bai, M., Roubos, E.W. Jenks, B.G.: Expression and characterization of the extracellular Ca(2+)-sensing receptor in melanotrope cells of *Xenopus laevis*. Endocrinology, 2003; 144: 2524-2533.

13. Wang, Y., Awumey, E.K., Chatterjee, P.K., Somasundaram, C., Bian, K., Rogers, K.V., Dunn, C., Bukoski, R.D.: Molecular cloning and characterization of a rat sensory nerve Ca2+-sensing receptor. Am. J. Physiol. Cell Physiol., 2003; 285: 64-75.

14. Ziegelstein, R.C., Xiong, Y., He, C., Hu, Q.: Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells. Biochem. Biophys. Res. Commun., 2006; 342: 153-163.

15. Lee, E.Y., Fukuda, Y., Kuwaki, T.: Cardiovascular neurons of the rat brain express extracellular calcium-sensing receptors. Auton. Neurosci., 2007; 135: 77-78.

16. Cheng, I., Qureshi, I., Chattopadhyay, N., Qureshi, A., Butters, R.R., Hall, A.E., Cima, R.R., Rogers, K.V., Hebert, S.C., Geibel, J.P., Brown, E.M., Soybel, D.I.: Expression of an extracellular calcium-sensing receptor in rat stomach. Gastroenterology, 1999; 116: 118-126.

17. Rasschaert, J., Malaisse, W.J.: Expression of the calcium-sensing receptor in pancreatic islet B-cells. Biochem. Biophys. Res. Commun., 1999; 264: 615-618.

18. Rácz, G.Z., Kittel, A., Riccardi, D., Case, R.M., Elliott, A.C., Varga, G.: Extracellular calcium sensing receptor in human pancreatic cells. Gut, 2002; 51: 705-711.

19. Kos, C.H., Karaplis, A.C., Peng, J.B., Hediger, M.A., Goltzman, D., Mohammad, K.S., Guise, T.A., Pollak, M.R.: The calcium-sensing receptor is required for normal calcium homeostasis independent of parathyroid hormone. J. Clin. Invest., 2003; 111: 1021-1028.

20. Smajilovic, S., Tfelt-Hansen, J.: Calcium acts as a first messenger through the calcium-sensing receptor in the cardiovascular system. Cardiovasc. Res., 2007; 75: 457-467.

21. Yarden, N., Lavelin, I., Genina, O., Hurwitz, S., Diaz, R., Brown, E.M., Pines, M.: Expression of calcium-sensing receptor gene by avian parathyroid gland in vivo: relationship to plasma calcium. Gen. Comp. Endocrinol., 2000; 117: 173-181.

22. Houillier, P., Nicolet-Barousse, L., Maruani, G., Paillard, M.: What keeps serum calcium levels stable? Joint Bone Spine, 2003; 70: 407-413.

23. Ramasamy, I.: Recent advances in physiological calcium homeostasis. Clin. Chem. Lab. Med., 2006; 44: 237-273.

24. Raue, F., Haag, C., Schulze, E., Frank-Raue, K.: The role of the extracellular calcium-sensing receptor in health and disease. Exp. Clin. Endocrinol. Diabetes, 2006; 114: 397-405.

25. Brown, E.M., Hebert, S.C.: Calcium-receptor-regulated parathyroid and renal function. Bone, 1997; 20: 303-309.

26. Chattopadhyay, N., Cheng, I., Rogers, K., Riccardi, D., Hall, A., Diaz, R., Hebert, S.C., Soybel, D.I., Brown, E.M.: Identification and localization of extracellular Ca2+-sensing receptor in rat intestine. Am. J. Physiol., 1998; 274: 122-130.

27. Houillier, P., Paillard, M.: Calcium-sensing receptor and renal cation handling. Nephrol. Dial. Transpl., 2003; 18: 2467-2470.

28. Chattopadhyay, N., Brown, E.M.: Role of calcium-sensing receptor in mineral ion metabolism and inherited disorders of calcium-sensing. Mol. Genet. Metab., 2006; 89: 189-202.

29. Liao, J., Schneider, A., Datta, N.S., McCauley, L.K.: Extracellular calcium as a candidate mediator of prostate cancer skeletal metastasis. Cancer Res., 2006; 66: 9065-9073.

30. Brown, E.M., Chattopadhyay, N., Vassilev, P.M., Hebert, S.C.: The calcium-sensing receptor (CaR) permits Ca^{2+} to function as a versatile extracellular first messenger. Recent Prog. Horm. Res., 1998; 53: 257-281.