# DISTRIBUTION OF CALCIUM SENSING RECEPTOR IN RATS: AN IMMUNOHISTOCHEMICAL STUDY

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**Objective.** To investigate the organ distribution of calcium sensing receptor (CaR) in rats by immunohistochemical method.

**Methods.** CaR was identified immunohistochemically in the rat tissues using specific anti-CaR antiserum raised in New Zealand white rabbits immunized with a conjugate of synthetic CaR peptide (186-204) with bovine serum albumin. Immunohistochemical analysis was performed by avidin-biotin complex method.

**Results.** CaR immunoreactivity was visualized in the central nervous system, anterior pituitary, gastric mucosa, small intestine and colon, Auerbach·s and Meissner·s gastric nervous branch, small intestine and colon, pancreas, adrenal medulla, kidney and testis. When using antiserum preincubated with synthetic CaR peptide (186-206) or kidney homogenates, no significant stain of kidney was detected.

**Conclusions.** The findings suggest that CaR is widely distributed and that the method used is valuable in studying the distribution of caR in rat.

**Key words:** Calcium sensing receptor – Immunohistochemistry – Organ distribution – Rat

Calcium ion is essential for vital body functions (Pietrobon et al. 1990; Brown 1991) and calcium sensing receptor (CaR) is a member of G-protein coupled receptor superfamily which has been cloned from bovine parathyroid and rat kidney (Brown et al. 1993; Aida et al. 1995; Garrett et al. 1995a; Ricardi et al. 1995). The binding of calcium to this receptor results in the activation of G-protein which further stimulates phospholipase C activity (Brown et al. 1993, 1995; Chattopadhyay et al. 1996). Moreover, it has been reported that sporadic hypoparathyroidism caused by *de novo* gain-of-function mutations of the $Ca^{2+}$ sensing receptor and CaR mutations also cause in familial benign hypercalcemia and neonatal hyperparathyroidism (Pearce et al. 1995, 1997; Pollak et al. 1996; De Luca et al. 1997; Kobayashi et al. 1997; Watanabe et al. 1998). Such precedent evidence indicates that this peptide involves calcium metabolism, neural and gastrointestinal functions (Chattopadhyay et al. 1996).

Thus, it would be of interest to investigate the precise distribution of CaR in rats, which has not yet been reported. We used an anti-CaR antibody which was raised in rabbits immunized with a conjugate of synthetic CaR peptide (186-209) to bovine serum albumin (BSA).

## Materials and Methods

**Animals.** Male Wistar rats weighing 250-280 g were obtained from the Shizuoka Animal Laboratory Co. Ltd. (Shizuoka, Japan) and housed in temperature (22 °C) and humidity (60 %) controlled room under 12 h illumination cycle. They were fed with laboratory chow and water *ad libitum*.

**Drugs.** BSA, glutaraldehyde, paraformaldehyde, sodium pentobarbital and Bouin·s solution were ob-

tained from Katayama Chem. Co. Ltd. (Osaka, Japan), all reagents being of analytical grade. Complete Freund's adjuvant was obtained from Wako Chemical Co. Ltd. (Osaka, Japan).

**Preparation of anti-CaR antibody.** Peptide corresponding to CaR (186-206) was synthesized with solid phase method using an automated peptide synthesizer, followed by purification with HPLC: LGLFYIPQVSYASSSRLLSNKNQY. Synthetic CaR (186-209) was conjugated on an equal weight basis to BSA by the method previously described for anti-GHRH antibody (MITSUMA et al. 1986), using glutaraldehyde.

New Zealand white rabbits weighing about 3 kg were obtained from Nakajima Animal Laboratory Co. (Gifu, Japan). They were immunized with the emulsion of one mg of this conjugate in one ml water in complete Freund's adjuvant (1:2, v/v) which was injected into foot pad at intervals of three weeks. Blood was drawn one week after each injection and the presence of anti-CaR was checked.

**Preparation of tissue for CaR estimation.** CaR contained fraction was obtained by the method of RUAT et al. (1995). The kidneys were removed from the rat and a pool of kidneys weighing 2 g was homogenized in 20 ml ice-cold solution containing 50mM Tris-HCl (pH 7.4), 1mM EDTA, aprotinin (10 μg/ml), benzamidine (60 μg/ml) and centrifuged at 100,000 x g for one hour. The pellet was resuspended in the above buffer to working concentration of 200 mg/original wet weight and used as CaR fraction.

**Perfusion method and immunohistochemical method.** The rats were anesthetized with intravenous administration of sodium pentobarbital (20 mg/kg) and transcardially perfused with 0.01 % glutaraldehyde and 4 % paraformaldehyde in Bouin's solution (pH 7.2). The brain, spinal cord, pituitary gland, lung, heart, kidney, liver, thyroid gland, parathyroid gland, stomach, small intestine, colon, adrenal gland, pancreas and testis were removed and post-fixed for an additional hour at 4 °C, then cut at 4 μm using a vibratome. Immunohistochemical treatment was performed by avidin-biotin complex (ABC) method, using Vecstatin kits (Vector Laboratories, Burlingame, CA).

The primary antibody was used after dilution (1:50). To confirm the specificity of anti-CaR antibody, the following methods were used in the kidney: 1. omission of the primary antiserum or second antiserum in the peroxidase-antiperoxidase technique, 2. reabsorption of the antiserum prior to the incubation of experimental tissues with synthetic peptide CaR (186-206) (1.0 mg/ml antiserum) or kidney homogenate containing CaR. Specific immunohistochemical stain could be not seen in any of these control paradigm (Fig. 1), 3. serial dilution of primary antiserum was used. Specific stain disappeared at 1:5000 dilution. the brain nuclei were determined using the map of PELLEGRINO et al. (1969).

## Results

As shown in Tab. 1 and Fig. 1, specific staining appeared in the central nervous system, anterior pi-

**Table 1**
**Distribution of calcium-sensing receptor in the rat central nervous system**

1. **Telencephalon**
   *Olfactory system:* anterior olfactory nucleus, olfactory tubercles
   *Amygdaloid and related areas:* cortical nuclei, central nuclei, medial nucleus
   *Cortex:* pyriform, frontal-orbital, frontal-parietal, entorhimal
   *Hippocampal formation:* cornus Ammonis, dentate gyrus, stratum pyramidale, subiculum
   *Septal region:* lateral septal nucleus, medial septal nucleus, nucleus striae terminalis, lateral preoptic area
   *Basal ganglia and related area:* globus pallidus, claustrum, nucleus basalis of Meynert, nucleus striae terminalis, endopiriform nucleus
2. **Diencephalon**
   *Thalamus:* perithalamic and subthalamic areas, centralmedial nucleus, paratential nucleus, ventromedial nucleus, renien nucleus, posterior nucleus
   *Hypothalamus:* paraventricular nucleus, arcuate nucleus, ventromedial nucleus, anterior hypothalamic area, lateral hypothalamic area, periventricular hypothalamic nucleus
3. **Mesencephalon**
   substantia nigra, ventral tegmental area, interpeduncular nuclear complex, posterior commissure, periaquaeductal gray
4. **Pons/Medulla**
   locus coeruleus, nucleus subcerulaeus, Rahe complex, central gray pons, trigeminal nucleus, suprafacial nucleus, amiguous nucleus, nucleus of the solitary tract, hypoglossal nucleus, lateral reticular nucleus, parvicellular reticular nucleus
5. **Cerebellum**
   cerebellar peduncles, cerebellar cortex, deep cerebellar nucleus
6. **Spinal cord**
   ventral horn, dorsal horn, lamina i, II and II
7. **Retina**

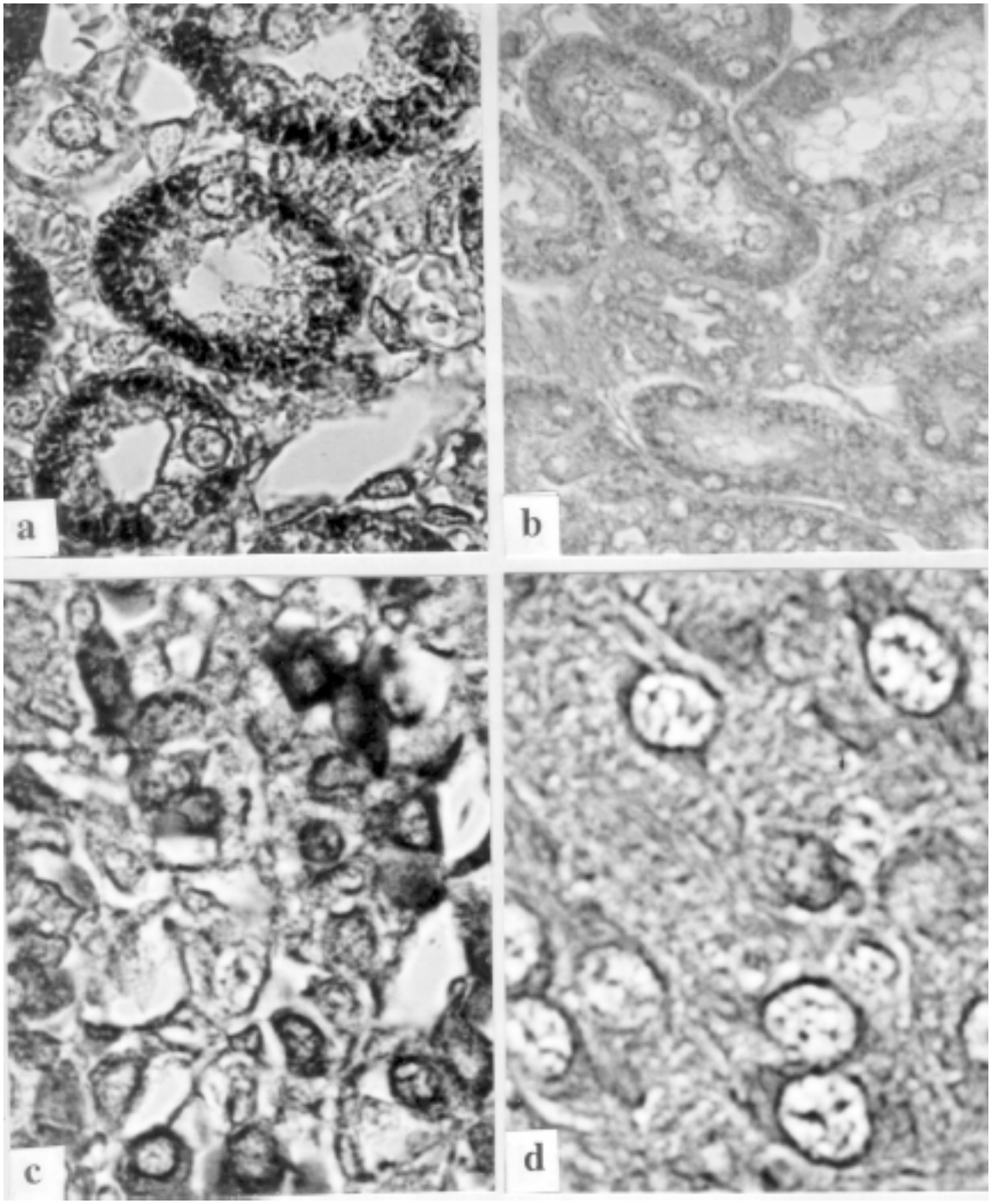


**Fig. l (upper pictures) Distribution of CaR in the kidney: a - positive staining in the kidney tubules (x 200); b - positive staining disappeared when antibody was applied prior to incubation with an excessive amount of CaR (x 200). (lower pictures) Distribution of CaR in the anterior pituitary (c) and cerebrum (d), x 200.**

tuitary, parathyroid, parafollicular cells of the thyroid, kidney, gastric mucosa, small intestine and colon, Auerbach's and Meissner's nervous branch of the stomach, small intestine and colon, adrenal medulla, pancreas, lung and retina, but not in the heart, liver and posterior pituitary. In the central nervous system, significant stain revealed neural pericarya, axons and dendrites.

## Discussion

The distribution of CaR immunoreactivity in the rat body was immunohistochemically investigated. Anti-CaR was raised in New Zealand white rabbits by repeated injection of a conjugate of synthetic peptide CaR (186-206) to BSA. It was found that this antibody is specific and the method used in this experiment can be used to detect the distribution of CaR in the rat. The present study clearly demonstrated that the CaR is widely distributed in the rat body. It has been reported that CaR mRNA was expressed in bovine parathyroid, cerebral cortex, cerebellum, kidney and thyroid. CaR mRNA was also expressed in rat thyroid parafollicular C-cells, testis, lung, ileum, large intestine, adrenal gland, central nervous system and pituitary (Brown et al. 1993; Garrett et al. 1995a; Ricardi et al. 1995; Ruat et al. 1995). The present study confirmed these findings on the protein level. Roger et al. (1995) reported that CaR mRNA levels in parathyroid glands and kidney of vitamin D-deficient rats were not regulated by plasma calcium or 1,25-dihydroxyvitamin $D^3$. In contrast, the treatment of rats with 1,25-dihydroxyvitamin $D^3$ *in vivo* resulted in an increase in the levels of CaR mRNA (Zohng et al. 1994). In dispersed bovine parathyroid cells, the loss of the ability to suppress parathyroid hormone release in responses to $Ca^{2+}$ was parallel by rapid reduction in CaR expression (Mithal et al. 1995). Moreover, mutation of CaR cDNA caused familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism (Pearce et al. 1995, 1997; Pollak et al. 1996; De Luca et al. 1997; Kobayashi et al. 1997; Watanabe et al. 1998). The present study demonstrated the CaR was found in the parafollicular cells of the thyroid which produce calcitonin and the presence of CaR in these cells indicates that calcitonin release may be mediated via CaR in C-cell (Garrett et al. 1995b; Freichel et al. 1996). These findings suggest that CaR play important role in calcium homeostasis.

The function of CaR in the brain is unknown at present. CaR regulates the activity of $Ca^{2+}$-permeable nonselective cation channels in hippocampal neurons and could modulate functions of these cells (Ye et al. 1996). Thus, CaR may regulate the learning and memory. The function of CaR in the pituitary, lung, gastrointestinal tract, testis and pancreas is unknown and remains to be elucidated.

In conclusion, the present findings suggest that CaR is widely distributed in the rat body and this method is useful to study the distribution of CaR.

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