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Calcium sensing receptor: Molecular cloning in rat and localization to nerve terminals

(parathyroid/thyroid hormone/cerebral blood vessel/kidney/pituitary)

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ABSTRACT **We have molecularly cloned a calcium sensing receptor (CaSR) from a rat striatal cDNA library. Rat CaSR displays 92% overall homology to its bovine counterpart with seven putative transmembrane domains characteristic of the superfamily of guanine nucleotide-binding proteins and significant homology with the metabotropic glutamate receptors. Northern blot analysis reveals two transcripts in thyroid, kidney, lung, ileum, and pituitary. In brain highest regional expression of the RNA occurs in the hypothalamus and the corpus striatum. Immunohistochemistry reveals discrete punctate localizations throughout the brain that appear to be associated with nerve terminals. No staining is evident in cell bodies of neurons or glia. Cerebral arteries display an intense network of CaSR immunoreactive fibers associated with vessel innervation. CaSR on nerve terminal membranes may regulate neurotransmitter disposition in response to Ca^{2+} levels in the synaptic space.**

Calcium iöns are crucial for a variety of neuronal functions. Neurotransmitter release is triggered by Ca^{2+} entry in nerve terminals, while Ca^{2+} release by the inositol 1,4,5-trisphosphate receptor or the ryanodine receptor regulates intracellular signal transduction. Numerous peripheral organs monitor extracellular Ca^{2+} levels to regulate the disposition of calcium in tissues such as the parathyroid gland, kidney, and bone (1). Recently, Brown *et al.* (2) cloned a Ca^{2+} sensing receptor (CaSR) from bovine parathyroid tissue. CaSR, a 120-kDa membrane protein, possesses a very large extracellular N-terminal domain that resembles metabotropic glutamate receptors (mGluRs) and seven transmembrane domains (TMs) as in guanosine triphosphate binding protein (G protein)-coupled receptors. When expressed in oocytes, the protein is activated by Ca^{2+} leading to the G-protein-dependent activation of phospholipase C. Point mutations in CaSR are linked to diseases that display abnormalities in blood Ca^{2+} disposition (3, 4). Northern blot analysis reveals expression of CaSR in several tissues other than the parathyroid gland, including brain (2).

In the present study, we have molecularly cloned the rat form of CaSR from a brain cDNA library.† With an antiserum to rat CaSR, we have localized CaSR selectively to nerve terminals in brain and blood vessels.

MATERIALS AND METHODS

Cloning and Sequencing of a Rat cDNA for CaSR. A rat striatal cDNA library constructed in λ ZAPII (Stratagene) was screened (1,000,000 phages) with a ^{32}P-labeled DNA (5) containing two overlapping oligodeoxynucleotides (5′-TTCCTCCGCACCATACCCAATGATGAACACC-AGGCCACGGCCATGGCTG and 5′-GCCCACCCAGTTC-CAGCGGAAGTACTCGATGATGTCAGCCATGGCCG) derived from the bovine CaSR sequence, i.e., within the putative hydrophobic domain located in the extracellular domain (2). Twenty positive clones were further plaque-purified and rescued plasmids [Bluescript KS(+)] containing cDNA inserts were sequenced by the fluorescent terminator method of cycle sequencing on an Applied Biosystems model 373a automated DNA sequencer. Sequencing of a 6.5-kb cDNA insert derived from clone SRCa33 indicated an open reading frame of 1079 aa beginning with an initiator consensus sequence methionine (AACGCTATGG) (6) starting from Met^1 (Fig. 1), with a nonsense codon (TGA) at position −18, and ending with a stop codon (TAA) at position 3238.

Northern Blot Analysis. RNAs or poly(A)$^+$ mRNAs from adult male Sprague–Dawley rats were prepared and subjected to Northern blot analysis as described (5) except that 50% (vol/vol) formamide was used in the prehybridization and in the hybridization buffers. A DNA probe ^{32}P-labeled by nick-translation and corresponding to the nucleotide sequence encoding aa 1–1079 (Fig. 1) was used. Blots were washed for two 20-min periods in 2× standard saline citrate (SSC)/0.1% SDS at 42°C and then in 0.2× SSC/0.1% SDS for two 20-min periods at 42°C, for two 15-min periods at 55°C, and for one 10-min period at 65°C. Blots were exposed to film for 4 days at −80°C.

Expression in Human Embryonic Kidney (HEK)-293 cells. A 3760-bp fragment containing the full-length coding sequence of the rat CaSR was excised from the 6.5-kb cDNA insert of the Bluescript plasmid, purified by Geneclean II (Bio 101), and ligated at the *Xho* I–*Xba* I sites of the expression vector pRK5. HEK-293 cells were transfected by using the Ca^{2+} phosphate procedure and cells were harvested for Western blot analysis.

Generation of Polyclonal Antisera to Rat CaSR. A synthetic peptide (KALAWHSSAYGPDQRAQ) based on the rat CaSR sequence (Fig. 1) was synthesized, conjugated to bovine serum albumin via glutaraldehyde, and injected in rabbits to raise antiserum (7). Antibodies were affinity-purified on an ovalbumin–CaSR peptide conjugate immobilized on CNBr-activated Sepharose.

Western Blot Analysis and Immunochemistry. Tissues or cells were homogenized in ice-cold 50 mM Tris·HCl, pH 7.4/1 mM EDTA/aprotinin (10 μg/ml)/leupeptin (10 μg/ml)/phenylmethylsulfonyl fluoride (100 μg/ml)/benzamidine (60 μg/ml) and centrifuged at 100,000 × *g* for 1 hr. Pellets were resuspended in buffer, and proteins were separated on a 7.5% polyacrylamide gel, transferred to immobilon-P membranes (Millipore), probed overnight with affinity-purified antibodies (2 μg/ml), and developed with enhanced chemiluminescence (Renaissance; DuPont/NEN). For preabsorption experi-

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Abbreviations: CaSR, calcium sensing receptor; G protein, guanosine triphosphate binding protein; GFAP, glial fibrillary acidic protein; mGluR, metabotropic glutamate receptor; TM, transmembrane domain.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U20289).

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MASYSCCLAL LALAWHSSAY GPDQRAQKKG DIILGGLFPI HFGVAAKDQD LKSRPESVEC   60
          SP
IRYNFRGFRW LQAMIFAIEE INSSPSLLPN MTLGYRIFDT CNTVSKALEA TLSFVAQNKI  120
                      *       *
DSLNLDEFCN CSEHIPSTIA VVGATGSGVS TAVANLLGLF YIPQVSYASS SRLLSNKNQY  180
          *
KSFLRTIPND EHQATAMADI IEYFRWNWVG TIAADDDYGR PGIEKFREEA EERDICIDFS  240
                     H
ELISQYSDEE EIQQVVEVIQ NSTAKVIVVF SSGPDLEPLI KEIVRRNITG RIWLASEAWA  300
                      *                     *
SSSLIAMPEY FHVVGGTIGF GLKAGQIPGF REFLQKVHPR KSVHNGFAKE FWEETFNCHL  360
QEGAKGPLPV DTFVRSHEEG GNRLLNSSTA FRPLCTGDEN INSVETPYMD YEHLRISYNV  420
                           *
YLAVYSIAHA LQDIYTCLPG RGLFTNGSCA DIKKVEAWQV LKHLRHLNFT NNMGEQVTFD  480
                           *
ECGDLVGNYS IINWHLSPED GSIVFKEVGY YNVYAKKGER LFINEEKILW SGFSREVPFS  540
          *
NCSRDCQAGT RKGIIEGEPT CCFECVECPD GEYSGETDAS ACDKCPDDFW SNENHTSCIA  600
*                                                      *
KEIEFLAWTE PFGIALTLFA VLGIFLTAFV LGVFIKFRNT PIVKATNREL SYLLLFSLLC  660
                          TM1                          TM2
CFSSSLFFIG EPQDWTCRLR QPAFGISFVL CISCILVKTN RVLLVFEAKI PTSFHRKWWG  720
                                TM3
LNLQFLLVFL CTFMQILICI IWLYTAPPSS YRNHELEDEI IFITCHEGSL MALGSLIGYT  780
          TM4                                                TM5
CLLAAICFFF AFKSRKLPEN FNEAKFITFS MLIFFIVWIS FIPAYASTYG KFVSAVEVIA  840
            "                      TM6
ILAASFGLLA CIFFNKVYII LFKPSRNTIE EVRSSTAAHA FKVAARATLR RPNISRKRSS  900
          TM7                                                  #
SLGGSTGSIP SSSISSKSNS EDRFPQPERQ KQQQPLSLTQ QEQQQQPLTLH PQQQQQPQQ  960
#
PRCKQKVIFG SGTVTFSLSF DEPQKNAMAH RNSMRQNSLE AQRSNDTLGRH QALLPLQCA 1020
DADSEMTIQE TGLQGPMVGD HQPEMESSDE MSPALVMSTS RSFVISGGGSS VTENVLHS  1079
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FIG. 1. Deduced amino acid sequence of the rat CaSR. The seven putative TMs (TM1–TM7), an additional hydrophobic domain (H), a consensus signal peptide sequence (SP), and stretches of acidic amino acids (E and D) are underlined. The symbols *, “, and # represent consensus sites for glycosylation and protein kinase C and A phosphorylation, respectively.

ments, the antibodies were preincubated overnight at 4°C with excess CaSR peptide (40 μg/ml).

Adult rats were perfused with 4% (wt/vol) freshly depolymerized paraformaldehyde in 0.1 M sodium phosphate (PB, pH 7.4). The brains were removed, postfixed for 2 hr at 4°C in 4% paraformaldehyde in PB before cryoprotection in 20% (vol/vol) glycerol in PB overnight. Sections were cut (20–40 μm) on a sliding microtome. Free-floating sections were transferred to 50 mM Tris-buffered saline (pH 7.4), permeabilized in 0.2% Triton X-100 for 30 min, blocked in 4% (vol/vol) normal goat serum for 30 min, and incubated overnight at 4°C with 2% normal goat serum/0.1% Triton X-100 containing one of the following antisera: affinity-purified CaSR antibodies (2–4 μg/ml), glial fibrillary acidic protein (GFAP) antibodies (1:5000 dilution, Dako), or neurofilament protein antibodies (SMI35, 1:20,000 dilution, Sternberger–Meyer, Jarrettsville, MD). Staining was visualized with an avidin–biotin-peroxidase system (Vectastain ABC kit, Vector Laboratories) with diaminobenzidine (0.01%) as a chromogen. For preabsorption experiments, CaSR antibodies were preincubated overnight at 4°C with excess CaSR peptide (200 μg/ml).

RESULTS

Molecular Cloning of Rat CaSR and Northern Blot Analysis. We cloned a cDNA containing an open reading frame encoding CaSR from a rat corpus striatum cDNA library by using a DNA probe derived from the bovine CaSR sequence (Fig. 1). The cloned protein of 1079 aa has a deduced estimated molecular mass of 120 kDa, resembling bovine CaSR (2). Rat CaSR has three domains: (*i*) a very long N-terminal portion, ≈600 aa that is presumably extracellular, begins with a putative signal peptide sequence (8), and has a hydrophobic segment; (*ii*) a seven TM zone delineating short intra- and extracytoplasmic loops; and (*iii*) a 250-aa C-terminal tail with potential sites for phosphorylation by cAMP-dependent protein kinase and a polyglutamine-rich region.

Rat CaSR has 92 or 95% amino acid identity with bovine (2) and human (GenBank accession no. X81086) CaSRs. The highest conservation of bovine, human, and rat sequences is in the central core of the protein displaying the putative TMs. The N-terminal extracellular region has a similarly high percent conservation, while the least conservation is in the C-terminal intracellular domain with ≈85% amino acid sequence identity (Fig. 2).

Rat CaSR has very little overall homology with other members of the G-protein-coupled receptor superfamily except for 20–25% sequence identity with mGluRs (9–13). The greatest similarity to the mGluR family is in TMs and throughout the N-terminal tail as indicated by the conservation of ≈20 Cys residues (Fig. 2). Glycoprotein hormone receptors (14, 15) also have long N-terminal domains but lack sequence resemblance to CaSR. The hydrophobic segment has 40–50% amino acid identity to the corresponding region in mGluRs but not in the $5HT_7$ serotonin receptor (16). Besides similarities to mGluR, the amino tail of CaSR also resembles bacterial periplasmic binding proteins (17).

Rat CaSR possesses 10 putative glycosylation sites concentrated in the N-terminal area. Two Cys residues (Cys^{677} and Cys^{765}) in the first and second extracellular loops correspond to similar residues in other G-protein-coupled receptors and are associated with a disulfide bridge. The third intracellular loop, an area usually associated with G proteins, is quite short and possesses a consensus sequence for protein kinase C phosphorylation. A missense mutation at this site in the human protein is possibly associated with abnormalities in extracellular calcium homeostasis (3). The rat C terminus has a 40-aa area in which half the amino acids are Gln. The extracellular domain possesses an unusually high number of acidic residues present as doublets or triplets (Fig. 1) that may be zones that bind Ca^{2+} (2). Such an area is also found in the second extracytoplasmic loop. CaSR does not possess any E-F hand motif classically associated with high-affinity Ca^{2+} binding in other proteins (18, 19).

Northern blot analysis revealed expression of CaSR in multiple rat tissues (Fig. 3). The thyroid, including parathyroid tissue, had a substantial amount of expression with bands at 8.5

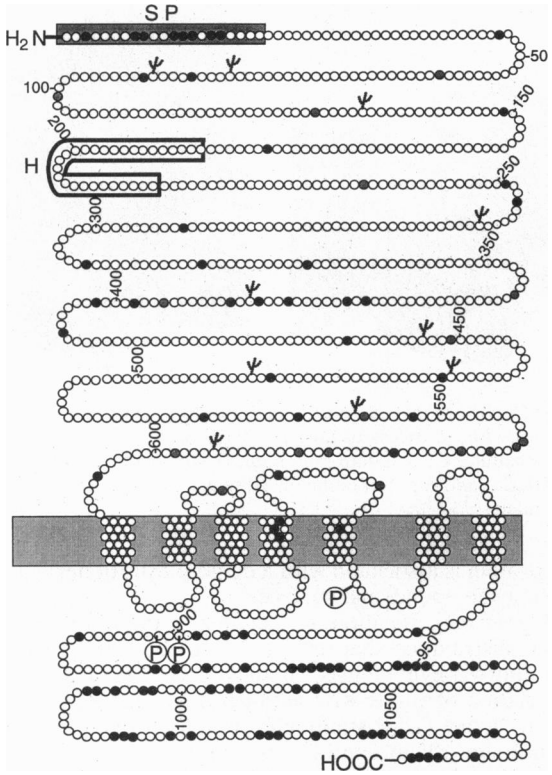

FIG. 2. Predicted topology of the rat CaSR. Amino acids that differ from the bovine or the human CaSRs are solid and conserved Cys residues with mGluRs are shaded. Consensus sites for glycosylation (ψ) and protein kinase A and C phosphorylation (P) are indicated. Amino acids are numbered in the N- and C-terminal tails. Other abbreviations are in Fig. 1.

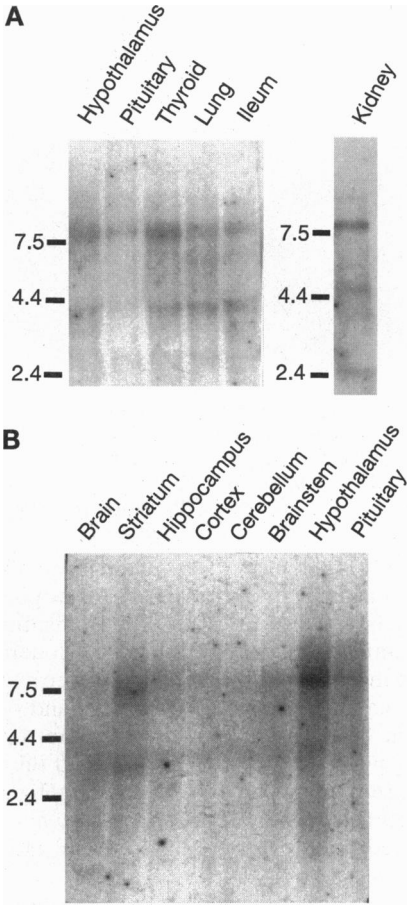

FIG. 3. Northern blot analysis of CaSR transcripts in rat tissues. RNAs (25 μg or 10 μg for pituitary) or poly(A)$^+$ mRNAs (kidney, 4 μg) were used. Molecular sizes (kb) are shown. *A* and *B* show results of two experiments.

and 3.8 kb, but hypothalamus, pituitary, lung, and ileum had comparable levels of CaSR mRNA. The adrenal gland also had a substantial amount of CaSR mRNA (data not shown). In contrast, in bovine tissue, Brown *et al.* (2) failed to detect CaSR mRNA in the lung, adrenal gland, and several parts of the gastrointestinal pathway including stomach, esophagus, and gall bladder. In the brain, we observed heterogeneity in CaSR mRNA levels with the highest level in the hypothalamus, next highest level in the corpus striatum, and a lower level in all other areas examined.

Whereas brain, pituitary, thyroid, lung, and ileum had a 3.8-kb band, kidney had a 5.5-kb band and the 8.5-kb band. In bovine tissue, the most prominent band is observed at 5.3 kb, though minor bands are evident at different sizes (2).

Localizations of CaSR Protein. We developed a rabbit polyclonal antiserum to a 17-aa peptide sequence in the N-terminal domain of rat CaSR, in an area displaying no sequence identity to any mGluRs. Immunoblot analysis with anti-CaSR antibody revealed an intense immunoreactive protein with an apparent molecular mass of 140 kDa in HEK-293 cells transiently expressing rat CaSR that was absent in untransfected cells (Fig. 4). Staining was not detected when the antiserum was preabsorbed with the antigen peptide (data not shown). A single immunoreactive band at 160 kDa was found in the corpus striatum and other brain regions (data not shown) with staining blocked in preabsorption experiments. Variations in molecular mass may reflect differential posttranslational modifications such as glycosylation.

Immunohistochemical staining of rat brain at low magnification revealed a moderate level of CaSR immunoreactivity throughout the brain with particular high levels and a distinct laminar distribution in the hippocampus and cerebellum (Figs. 5 *A* and *B* and 6 *A*, *D*, and *E*). In the cerebellum, the most intense staining was in a band coextensive with the Purkinje cell layer, with substantial staining in the granule cell layer and deep nuclei but negligible staining in the molecular layer (Figs. 5*A* and 6*A*). In the hippocampal formation, intense immunoreactivity was in the stratum moleculare and the stratum oriens of CA1 to CA3 with moderate staining in the dentate hilus. Immunoreactivity in cerebral cortex was more prominent in supragranular layers I–III and in layer II of cingulate cortex (Fig. 5 *A* and *B*). Very high levels of staining in the olfactory bulb were prominent in the glomeruli. Immunoreactivity was present in the neuropil of the external plexiform layer and in the lateral olfactory tract, which mainly represent fibers emerg-

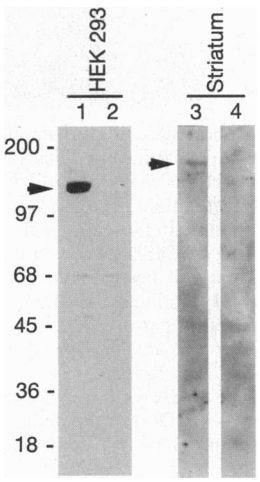

FIG. 4. Immunoblot analysis of membrane preparations from rat striatum and CaSR-expressing HEK-293 cells. Proteins from HEK-293 cells (25 μg) expressing CaSRs (lane 1) or not (lane 2) or from rat striatal membrane preparations (150 μg, lanes 3 and 4) were used. Preabsorption of the antibodies with an excess of the antigen peptide (40 μg/ml) completely blocked the striatal immunoreactive band (lane 4). Molecular sizes are indicated in kDa.

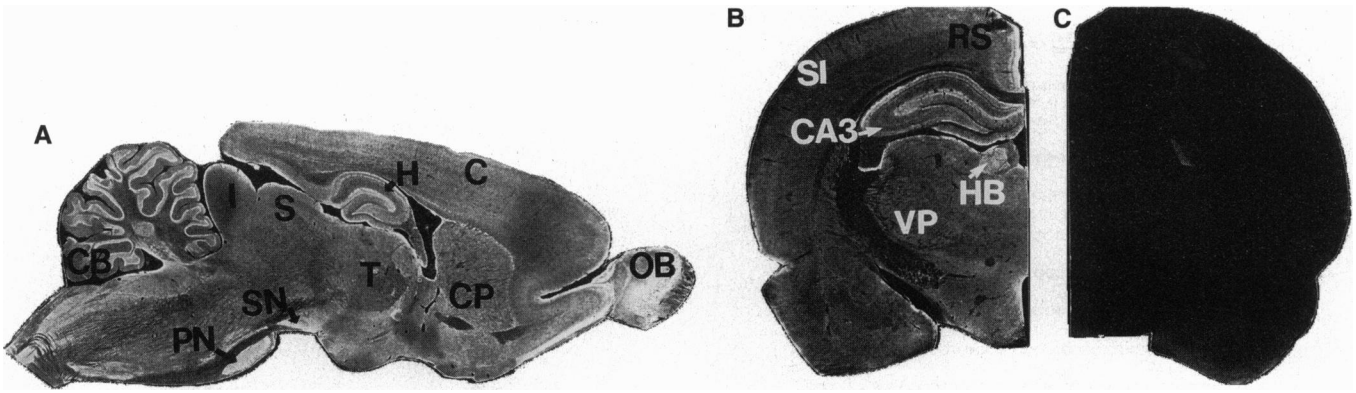

FIG. 5. Immunohistochemical localization of CaSR in rat brain sections. Saggital (*A*) or coronal (*B* and *C*) sections (40 μm) were stained with affinity-purified antibodies against rat CaSR (*A* and *B*). Preabsorption of antibodies with an excess of antigen (*C*) abolished immunostaining. Immunoreactive structures appear white in these images. C, cerebral cortex; CB, cerebellum; CP, caudate–putamen; H, hippocampus; HB, lateral habenula; OB, olfactory bulb; PN, pontine nuclei; RS, retrosplenial cortex; I and S, inferior and superior colliculi; SI, sensory cortex; SN, substantia nigra pars reticulata; T, thalamus; VP, ventral posterolateral and medial thalamic nuclei.

ing from the mitral and tufted cells. Staining was also observed in the substantia nigra pars reticulata and in the pontine nuclei. The lateral habenula had pronounced staining, but there was negligible staining in the medial habenula. Moderate staining was observed throughout the caudate–putamen and associated structures, such as the nucleus accumbens and olfactory tubercle, and in thalamic nuclei and the ventral hypothalamic area. The ependymal zone of the lateral and third ventricles showed intense immunoreactivity (Fig. 5*B*). Higher magnification showed that the ependymal cells were not labeled but were decorated by intensely stained puncta, indicating that CaSR protein is associated with a dense plexus of nerve fibers located at the ependymal surface.

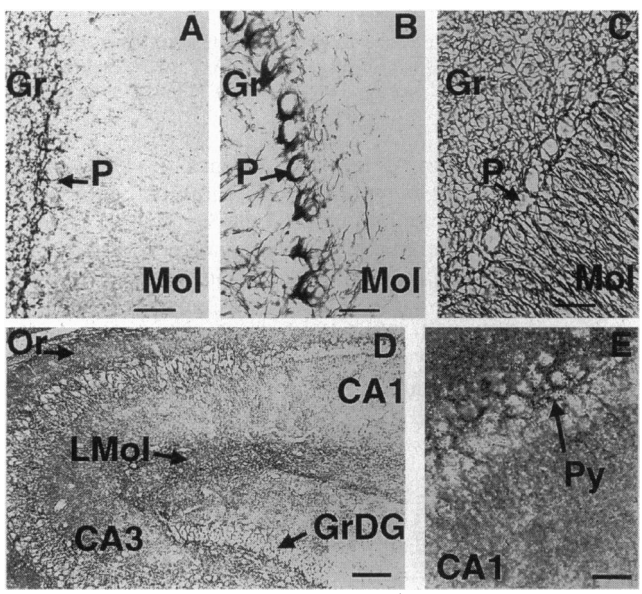

FIG. 6. Immunoreactivity of CaSR, phosphorylated neurofilament, and GFAP in rat cerebellum and hippocampus. Adjacent thick (20 μm) saggital (*A–C*) or coronal (*D* and *E*) sections were stained with antibodies against CaSR (*A, D,* and *E*), neurofilament protein (*B*), or GFAP (*C*). Dark areas represent positive staining in these bright-field images. In the cerebellum, punctate staining of CaSR occurs in the granule cell layer (Gr) and in the Purkinje cell layer around the Purkinje cell (P), reflecting processes of basket cells as indicated by neurofilament protein staining, but in a different pattern than astrocytic GFAP staining. In the hippocampus (*D* and *E*), punctate immunoreactivity of CaSR is abundant in stratum lacunosum molecular (LMol) and stratum oriens (Or) of CA1 to CA3 and is detected around pyramidal cells (Py) and granule cells (GrDG) of the dentate gyrus. Mol, molecular layer of the cerebellum. (Bars: *A–C* and *E*, 50 μm; *D*, 250 μm.)

At higher magnification, all labeling in the brain was in a punctate distribution that reflects nerve fibers and terminals. No staining was evident in cell bodies of neurons or glia. A lack of association of CaSR with astrocytes is emphasized by our failure to detect CaSR staining in primary astrocytic cultures (data not shown). In contrast, we observe substantial CaSR staining in primary rat cerebral cortical neuronal cultures (M.R., R. Ratan, L. Hester, and S.H.S., unpublished observations). Association with nerve terminals was especially notable in the cerebellum, hippocampus (Fig. 6*A, D,* and *E*), and cerebral cortex (data not shown). Immunoreactivity of fine axons reveals "baskets" surrounding the basal portion of the Purkinje cells reflecting terminals of basket cells, while Purkinje cells themselves are not stained. The highest density of stained basket axons simulates the pinceau surrounding the Purkinje cell initial segment (20). Very little staining was seen in the molecular layer, while a punctate network in the granule cell layer stained intensely. Immunostaining for phosphorylated neurofilament, which labels axons and terminals, revealed the same basket formation surrounding Purkinje cells with a network in the granule cell layer (Fig. 6*B*) but much less staining in the molecular layer. In contrast, the astrocytic marker GFAP displayed a very different pattern of staining that was particularly dense in the molecular layer (Fig. 6*C*).

In the hippocampus, pyramidal cell bodies themselves were not immunoreactive but were surrounded by intensely stained puncta in CA1 to CA3 (Fig. 6 *D* and *E*). The stratum radiatum of the hippocampus and the granule cell layer of the dentate gyrus was unlabeled. Immunostaining in the hippocampus associated with synaptic terminals was concentrated in three zones associated with pyramidal cells, i.e., basal dendrites, cell bodies, and apical dendrites.

Cerebral arteries displayed substantial CaSR staining in a network of branching nerve fibers (Fig. 7*A*) This pattern resembles neuronal nitric oxide synthase, which occurs in axons innervating cerebral vessels (21, 22). GFAP staining displayed a markedly different pattern (Fig. 7*C*). All cerebral vessels examined, including large, medium, and small vessels displayed the same pattern of CaSR staining (data not shown). Specific CaSR staining was eliminated by preabsorption of the antibody with the CaSR peptide (Figs. 5*C* and 7*B*) but not by an unrelated peptide used at the same concentration (data not shown). The signal was absent from sections when CaSR antibody was omitted.

DISCUSSION

The rat form of CaSR cloned in this study differs from bovine and human CaSR minimally in amino acid sequence. However,

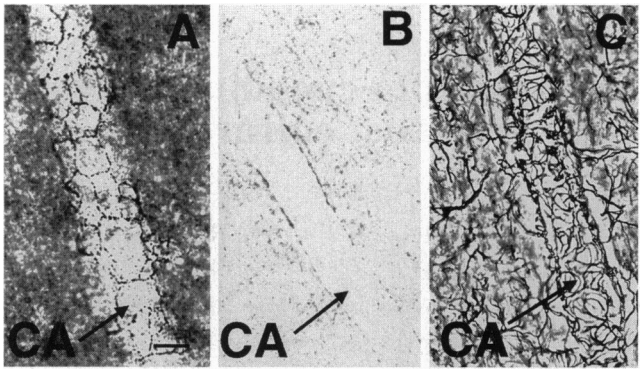

FIG. 7. Comparison of the CaSR and GFAP immunoreactivities on cerebral blood vessels. Prominent networks of CaSR immunoreactivity (*A*) surround a cerebral blood vessel and differ markedly from astrocytic GFAP-positive projections (*C*) obtained in an adjacent section (20 μm thick). Preabsorption of antibodies with an excess of antigen abolished CaSR staining (*B*). Dark areas represent positive staining in these bright-field images. (Bar = 50 μm.)

there are substantial differences in mRNA size, with the predominent form of CaSR in rat being 8.5 kb and in bovine tissue being 5.5 kb. The sizes of the different transcripts evident in this study are compatible with a protein of 1079 aa. By using poly(A)$^+$ mRNA, we have shown multiple transcripts of rat CaSR in brain and peripheral tissues (data not shown). Expression of these transcripts could reflect distinct 5′ starting sites or distinct 3′ polyadenylylation sites, alternatively spliced forms of CaSR, or homologous gene transcripts.

Though CaSR was discovered as a Ca^{2+} sensor, the protein is also activated to a lesser extent by Mg^{2+} (2). The Ca^{2+} sensor of the parathyroid gland responds to other polyvalent cations including drugs such as neomycin (23-25). The extracellular domains of CaSR and mGluRs resemble bacterial periplasmic proteins that detect nutrients such as sugars, amino acids, and a variety of ions (26). Perhaps CaSR in some tissues physiologically responds to substances other than Ca^{2+}.

The nerve terminal localization of CaSR in brain and blood vessels contrasts with parathyroid CaSR on the plasma membrane of the chief cells where CaSR detects serum Ca^{2+}. Ca^{2+} homeostasis is also regulated by vitamin D-dependent absorption through intestinal epithelium, suggesting that ileal CaSR influences Ca^{2+} absorption. In the kidney, CaSR presumably detects blood Ca^{2+} to modulate Ca^{2+} reabsorption. The high density of CaSR in the ependymal zone, which is in close contact with ventricular fluid, suggests that CaSR in this area responds to changes in ventricular Ca^{2+} concentration.

How might CaSR regulate nerve terminal activity in brain and blood vessels? Ca^{2+} influx into nerve terminals upon neuronal depolarization triggers neurotransmitter release. This event presumably causes local changes in the Ca^{2+} concentration of the synaptic cleft. CaSR may detect such changes and regulate nerve terminal responses to them. Global brain function is highly sensitive to serum Ca^{2+} levels, with major alterations in cognition associated with substantial increases or decreases in serum Ca^{2+}. Conceivably, CaSR in nerve fibers covering the ependymal layer participates in homeostatic mechanisms to deal with such alterations reflected in ventricular fluid Ca^{2+} levels.

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