Extracellular Calcium-sensing Receptor Is Expressed in Rat Hepatocytes

COUPLING TO INTRACELLULAR CALCIUM MOBILIZATION AND STIMULATION OF BILE FLOW

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Liver cells respond to changes in Ca\(^{2+}\). The hepatic functions affected include bile secretion, metabolic activity, liver regeneration, and the response to xenobiotics. In the present study, we demonstrate the presence, in the liver, of the extracellular calcium-sensing receptor (CASR), described previously in the parathyroid and thyroid glands and kidney. CASR mRNA was specifically expressed in hepatocytes and was absent in non-parenchymal liver cells (stellate, endothelial, and Kupffer cells). Western blot analysis using a specific CASR antibody showed staining in both whole liver and hepatocyte extracts. Immunohistochemistry and in situ hybridization of rat liver sections showed expression of CASR protein and mRNA by a subset of hepatocytes. The known agonists of the CASR, gadolinium (Gd\(^{3+}\); 0.5–3.0 mM) and spermine (1.25–20 mM), in the absence of Ca\(^{2+}\), elicited dose-related increases in Ca\(^{2+}\) in isolated rat hepatocytes loaded with Fura-2/acetoxymethyl ester. There was a greatly attenuated response to a second challenge with either agonist. The response was also abrogated when inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive calcium pools had been depleted by pretreatment with either thapsigargin or phenylephrine, an \(\alpha_1\)-adrenergic receptor agonist known to mobilize Ca\(^{2+}\) from IP\(_3\)-sensitive pools. Addition of the deschloro-phenylalkylamine compound, NPS R-467, but not the S enantiomer, NPS S-467, increased the sensitivity of the Ca\(^{2+}\)i mobilization response to 1.25 mM spermine. Bile flow ceased after Ca\(^{2+}\)o withdrawal, and its recovery was enhanced by spermine in isolated perfused liver preparations. The CASR agonists Ca\(^{2+}\) and Gd\(^{3+}\) increased bile flow, and the response to a submaximal Ca\(^{2+}\) concentration was enhanced by NPS R-467 but not the S compound. Thus, the data indicate that rat hepatocytes harbor a CASR capable of mobilizing Ca\(^{2+}\) from IP\(_3\)-sensitive stores and that activation of the CASR stimulates bile flow.

A calcium-sensing receptor (CASR) expressed on the cell surface of parathyroid, thyroid (calcitonin-secreting C cells), and kidney tubule cells senses changes in the extracellular calcium level ([Ca\(^{2+}\)]) within the normal circulating range in vivo within the normal circulating range in vivo (1). The modulation of cellular signaling pathways thereby brought about affects hormone secretion and renal electrolyte handling. The CASR plays a critical role in coordinating hormonally regulated systemic calcium homeostasis, and this is emphasized by the fact that naturally occurring loss or gain of function mutations in the CASR gene on human chromosome 3q13–21 (2) cause inherited hypercalcaemic or hypocalcaemic disorders, respectively (3–8). In addition, there is an association between serum calcium concentrations in the normal Caucasian population and a common polymorphism in the C-terminal tail of the CASR (9). The CASR is a G protein-coupled receptor, and its activation can lead to cell signaling via more than one pathway. Coupling to a \(G_{\text{q/11}}\) type protein activates phospholipase C effecting membrane phosphatidyl inositol bisphosphate turnover with production of inositol 1,4,5-trisphosphate (IP\(_3\)) and subsequent mobilization of Ca\(^{2+}\) from intracellular stores, as well as production of diacylglycerol and activation of protein kinase C (10, 11). Activation of the CASR leads to reduced production of intracellular CAMP in parathyroid cells (12) and cells of the nephron, for example, distal convoluted tubule cells (13). This might occur via coupling to the pertussis toxin-sensitive G\(_i\)-protein, which inhibits the adenyl cyclase enzyme, or via an indirect mechanism involving arachidonic acid metabolites (14).

With respect to ligand specificity the CASR is promiscuous, being responsive not only to divalent cations Ca\(^{2+}\) and Mg\(^{2+}\) but also trivalent cations such as the transition metal, gadolinium (Gd\(^{3+}\)) (15) and polycationic molecules like spermine (16). It has been proposed that the CASR could function as the physiological receptor for Mg\(^{2+}\) and polycations such as spermine, at certain sites in the body. Changes in ionic strength are also able to modulate CASR activation (17). In addition, certain phenylalkylamine compounds and their deschloro derivatives selectively potentiate the responsiveness of the CASR to its ligands by acting as positive allosteric modulators (18). Such compounds, so called calcimimetics, if of sufficient selectivity and potency, have enormous potential for the pharmacological modulation of PTH secretion and the medical management of hyperparathyroid states (19–21).

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‡‡ The abbreviations used are: CASR, Ca\(^{2+}\)-sensing receptor; RT, reverse transcriptase; PCR, polymerase chain reaction; IP\(_3\), inositol 1,4,5-trisphosphate; bp, base pair(s); AM, acetoxymethyl ester; FHH, familial hypocalciuria hypercalcaemia.
Although the CASR is highly expressed in tissues important for regulating the [Ca\(^\text{2+}\)]\(_\text{i}\) including parathyroid, thyroid, and kidney, it is also found at lower levels in many other tissues not known to play a role in calcium homeostasis (22). These latter tissues include regions of the brain such as hippocampus and pituitary, lung, and keratinocytes. For many of these, the physiological role of the CASR is not yet clear, and it may be that the CASR senses endogenous ligands other than Ca\(^{2+}\) at these particular sites.

With respect to the gastrointestinal tract and associated organs, the CASR is expressed in cells within the small and large intestines (23, 24), gastrin-secreting cells of the gastric antrum (25), different epithelial cells of gastric mucosa and enteric nerve regions (26), and acinar cells and interlobular ducts of the pancreas (27). However, CASR expression has not been studied in the liver. With respect to calcium homeostasis, the liver plays an important role in the uptake and metabolism of intact PTH, and this occurs specifically in Kupffer cells (28). PTH binding occurs on hepatocytes and sinusoidal cells and is linked in the former cells to adenyl cyclase (29, 30). At one time the hepatic metabolism of PTH was suggested to be [Ca\(^{2+}\)]\(_i\)-regulated, although this is now not thought to be the case (31). The liver is also the site of the C-25-hydroxylation of vitamin D, which is a prerequisite step for the final production of the hormonally active, 1,25-dihydroxylated metabolite. The 25-hydroxylase step has not yet been fully investigated with respect to regulation by [Ca\(^{2+}\)]\(_i\) (32).

However, liver cells do respond in a variety of ways to changes in [Ca\(^{2+}\)]\(_i\), and certain liver functions may be compromised when serum calcium levels rise or fall above or below the normal range. These functions include bile secretion, metabolic activity, liver regeneration, and resistance to xenobiotics. In the present study, we demonstrate the presence of the CASR in the liver. Its expression is diffuse throughout the hepatic acinus, the functionally active receptor mobilizes [Ca\(^{2+}\)]\(_i\) from IP\(_3\)-sensitive pools, and CASR agonists stimulate bile flow in the perfused rat liver.

**Experimental Procedures**

**Tissues**—Protocols for obtaining rat tissues were approved by local animal ethics committees. Tissues for RNA and Western blot analysis were flash frozen and stored at \(-80^\circ\text{C}\).

**Reverse Transcription-Polymerase Chain Reaction Analysis of the CASR**—Total RNA was extracted from tissues that were homogenized using a Polytron and from cells using TRIzol (Life Technologies, Inc.). RT-PCR was carried out as follows. First-strand cDNA was made by reverse transcribing 3 \(\mu\)l of DNase I-treated total RNA with a recombinant superscript II RNase H (Life Technologies, Inc.) using oligo(dT)\(_{15-18}\) (Amersham Pharmacia Biotech) in a total volume of 20 \(\mu\)l as described (33). Five microliters of the RT reaction were electrophoresed through 4–12% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, blocked with 5% dried milk powder in TBST for 3 h, and incubated with a CASR antibody. The mouse monoclonal antibody (ADD) was raised against a peptide comprising residues 214–236 of the extracellular domain of the CASR, which are completely conserved between human, bovine and rat. This antibody was kindly provided by Drs. P. K. Goldsmith and A. M. Spiegel (National Institutes of Health, Bethesda, MD) and K. V. Rogers (NPS Pharmaceuticals, Salt Lake City, UT). The specificity of this antibody for the parathyroid/kindey CASR has been previously well documented (13, 34). The antiserum was immunoaffinity purified to reduce the nonspecific binding. As a control, immunoblotting was carried out as described above with the antibody preadsorbed for 1 h with the peptide (10 \(\mu\)g/ml) against which it was raised. Antibody-antigen complexes were detected by chemiluminescence using the Lumi-glo kit (Life Technologies, Inc.).

**Immunohistochemistry of the CASR**—12-week-old female Harlan Sprague-Dawley rats (Charles River, St. Constance, Canada) were sacrificed by CO\(_2\) inhalation, and tissues were removed and fixed in 4% paraformaldehyde, 0.2% glutaraldehyde overnight at 4 \(^\circ\text{C}\). Specimens were dehydrated with an increasing concentration of ethanol before polymerization. 5-\(\mu\)m sections were mounted on Superfrost slides (Fisher, Nepean, Canada). Sections were post-fixed in 4% paraformaldehyde for 15 min, followed by a 10-min incubation in 10 \(\mu\)g/ml proteinase K (Life Technologies, Inc.) at room temperature to retrieve fixation-concealed antigens. After stabilization in 4% paraformaldehyde for 15 min, sections were incubated with the CASR monoclonal antibody (described above under Western bloting) at a dilution of 1:750 in 1% bovine serum albumin in phosphate-buffered saline overnight at 4 \(^\circ\text{C}\). After rinsing with phosphate-buffered saline, staining was done with the VECTASTAIN ABC kit (Vector Labs, Burlington, Canada) according to the manufacturer’s directions. As a control, some sections were treated with primary antibody that had been preadsorbed overnight at 4 \(^\circ\text{C}\) with the peptide (10 \(\mu\)g/ml) against which it had been raised.

In Situ Hybridization—In situ hybridization was carried out using biotin-labeled sense and antisense riboprobes and the Genepoint CSA kit (Dako Diagnostics, Mississauga, Canada). Labeled riboprobes were prepared from a plasmid containing a 509-bp insert corresponding to the rat CASR (13) using T3 and T7 RNA polymerases (Life Technologies, Inc.) and a nucleotide labeling mix containing biotin-16-UTP (Roche Molecular Biochemicals). Tissue sections were deparaffinized and rehydrated in a standard xylene and alcohol series and in situ hybridization was performed according to the manufacturer’s directions. Briefly, slides were heat in target retrieval solution and proteinase K to reveal hidden antibodies. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide in methanol. Sections were hybridized for 2 h at 50 \(^\circ\text{C}\) with 5 ng/ml biotin-labeled riboep in the supplied hybridization buffer at 37 \(^\circ\text{C}\). Sections were then washed at 50 \(^\circ\text{C}\) for 30 min prior to a 15 min wash at 4 \(^\circ\text{C}\). Sections were treated to successive incubations in primary streptavidin-horseradish peroxidase, biotinyl-tiamylamide solution, and secondary streptavidin-horseradish peroxidase (Dako) for 15 min each at room temperature. Color was developed with the supplied diaminobenidine diluted as directed (DAKO), and sections were counterstained with Carazzi’s hematoxylin prior to dehydration and mounting.

**Isolation of Nonparenchymal Liver Cells**—Sinusoidal cells were isolated from livers of nonfasting rats by the method of Knook and Sleyter (35) with the following modifications. After Nicodenz density gradient, cells were washed in Gey’s balanced salt solution (GBSS), pH 7.4, at 4 \(^\circ\text{C}\), resuspended, and introduced in a type J2-21M centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-6B elutriation rotor and a Sanderson chamber. While being centrifuged at 2500 rpm for 15 min, cells were washed out at pump flows of 13, 23, and 42 ml/min to collect stellate, endothelial, and Kupffer cells respectively using GBSS, pH 7.4 at 4 \(^\circ\text{C}\). Stellate, endothelial, and Kupffer cells were centrifuged and counted, and viability was evaluated. Sinusoidal cells had a viability greater than 95% and were free of hepatocytes.

**Isolation and Primary Culture of Hepatocytes**—Hepatocytes were obtained from livers of rats as described previously (36). The freshly isolated cells were suspended in William E medium containing 1% albumin, 0.5 mM glucose, sequentially filtered through 250- and 74-\(\mu\)m filters, and centrifuged at 250 rpm for 3 min. The freshly isolated hepatocytes were then equilibrated in William E medium (Life Technologies, Inc.). Cell viability was evaluated by Trypan blue exclusion, and those cells showing greater than 85% were taken for further study.
of additional Ca\textsuperscript{2+} conditions in which a submaximal flow was maintained, and the effects are according to GenBank\textsuperscript{TM} accession number U20289 and indicate the initiation codon, ATG, the start of each exon, and the stop codon, TAA. The positions of the primers used are indicated, and their sequences are given in Table I. The PCR products (products 1–5) generated are shown.

**Rat Bile Duct and Mouse Biliary Cell Line—**Rat whole bile duct was harvested. The mouse biliary cell line (BDC) was kindly provided by Dr. Emile Levy (Hôpital Ste-Justine, Montreal, Canada) and was cultured as described previously (37).

**Intracellular Calcium Measurements—**Hepatocytes were plated at a density of 3.5–5 \times 10\textsuperscript{5} cells/ml onto collagen-coated coverslips in Williams E medium containing 25 mM bicarbonate, 1% bovine serum albumin, at pH 7.4, 37 °C in a 5% CO\textsubscript{2} atmosphere. After incubation for 60 min cells were loaded for 30 min at 20 °C with the fluorescent probe Fuura-2/AM (2 \mu M) (Molecular Probes Inc., Eugene, OR) in bicarbonate-free Williams E medium supplemented with 0.25% fetal bovine serum and 1% bovine serum albumin. Dye-loaded cells were then transferred in a 100-ml plastic chamber to the stage of an inverted microscope (Diaphot, Nikon Corp., Tokyo, Japan) equipped for epifluorescence measurement. Cells were superfused at a rate of 3.6 ml/min, pH 7.4, at 32 °C in a 10 mM Krebs buffer containing 120 mM NaCl, 4.8 mM KCl, 5.5 mM glucose, 1.2 mM MgCl\textsubscript{2}, 0.2 mM EDTA for the gadolinium (0.5–3 mM) studies, and 10 mM Hepes buffer containing 120 mM NaCl, 4.8 mM KCl, 5.5 mM glucose, 1.2 mM MgSO\textsubscript{4}, 0.2 mM EDTA for the spermine (1–20 mM) studies. Phenylephrine and thapsigargin were used at a concentration of 5 \mu M.

Calcium measurements were made as described previously (38). Briefly, fluorescence signals from single hepatocytes were obtained with an MCID dual excitation spectrofluorometer system (Imaging Research Inc., St. Catharines, Canada). Excitation wavelengths were 340 and 380 nm, and fluorescence emission was measured at 505 nm. A refrigerated camera (Hamamatsu Photonics C4880, Hamamatsu City, Japan) was used as the imaging device. Output from the fluorometer was digitized, ratioed, and computer analyzed. Intracellular dye calibration was performed in situ by perfusion of 10 \mu M ionomycin in a solution containing 4 mM EGTA (R\textsubscript{max}) or 4 mM CaCl\textsubscript{2} (R\textsubscript{max}). After correction for sample autofluorescence, signal ratios (F\textsubscript{exc}/F\textsubscript{exc}) were transformed into [Ca\textsuperscript{2+}], as previously reported (39). The presence of nonhydrolyzable dye was periodically verified by quenching with 2 mM MnCl\textsubscript{2} and found to be negligible when compared with autofluorescence.

**Measurement of Bile Flow in Isolated Perfused Rat Liver—**To investigate the involvement of the CASR in bile secretion, bile flow was monitored in the isolated-perfused rat liver either (1) following a period of cholestasis induced by calcium deprivation in vitro or (2) under conditions in which a submaximal flow was maintained, and the effects of additional Ca\textsuperscript{2+}, Gd\textsuperscript{3+} or NPS 467 compounds were tested. Rats were anesthetized with forane, and the liver was perfused in situ as described previously (40) using a perfusion apparatus (MX Ambec Perfusion, MX International Inc., Aurora, CO). Briefly, the common bile duct was cannulated using PE-10 tubing (Clay-Adams, Parsippany, NJ), and the bile was collected throughout the experiment. The perfusion medium consisted of a 10 mM Hepes buffer, pH 7.4, saturated with a mixture of O\textsubscript{2}/CO\textsubscript{2} (95:5, v/v) that contained 120 mM NaCl, 4.8 mM KCl, 25 mM NaHCO\textsubscript{3}, 1.2 mM MgSO\textsubscript{4}, H\textsubscript{2}O, 21.4 \mu M taurocholic acid, and 100 mg/100 ml glucose. Experiment 1 was done in Ca\textsuperscript{2+}-free perfusate or in perfusate containing 1.25 mM Ca\textsuperscript{2+} and 0, 0.25 or 2.5 mM spermine. Experiment 2 was done in perfusate containing 0.5 mM Ca\textsuperscript{2+} (base-line conditions) throughout or at 20 min changed to 1.25 mM Ca\textsuperscript{2+}, 0.5 mM Gd\textsuperscript{3+}, 0.5 mM Ca\textsuperscript{2+} + 1 \mu M NPS R-467, or 0.5 mM Ca\textsuperscript{2+} + 1 \mu M NPS S-467 (0.5 mM MgSO\textsubscript{4} was used throughout). The liver remained in situ throughout the experiments, and its temperature was maintained at 37 °C. The perfusate was recirculated with a Masterflex pump (Cole Palmer Instrument Co., Chicago, IL) through a stainless steel filter. A pressure regulator was used to damp down pulsatile flow. The perfusion rate through the liver was set at 30 ml/min. In each preparation, perfusate Ca\textsuperscript{2+} measurements were done using a ICA2 ionic calcium analyzer (Radiometer, Copenhagen, Denmark).

**Statistical Analysis—**Data are presented as the means ± S.E. Significant differences between grouped means were determined by Tukey’s analysis of variance or by Student’s t test (41) where appropriate.

**RESULTS**

The CASR Gene Is Expressed in Liver—Expression of CASR mRNA was examined using semi-quantitative RT-PCR. A cDNA fragment of 367 bp corresponding to the CASR mRNA sequence encoded by part of exons 2 and 3 was amplified using forward primer r2BF and r3AR (Fig. 1 and Table I). Aliquots of the PCR reaction were taken after 21, 24, 28, and 32 cycles for CASR and 18, 21, 24, and 28 cycles for GAPDH and electro-phoresed through ethidium bromide-stained gels. Fig. 2 shows...
that a low level of CASR mRNA expression was observed in
total liver and hepatocytes compared with the parathyroid,
thyroid, and kidney. The series of PCR products 1, 2, 4, and 5,
which together comprise the entire coding region of rat CASR
mRNA, were amplified from rat liver cDNA, subcloned into
plasmid vectors, and sequenced. The derived sequence was
examined, and the PCR reactions were electrophoresed through
ethidium bromide-stained gels.

**FIG. 2.** The CASR gene is expressed in rat liver. Complementary DNA was
synthesized from total RNA and used as template for PCR amplification. A cDNA
of 367 bp corresponding to CASR mRNA encoded by parts of exons 2 and 3 was
amplified using primers r2BF and r3AR (Fig. 1 and Table I). A cDNA of 469 bp
comprising the entire coding region of rat CASR mRNA was also amplified. Aliquots were
taken at 21, 24, 28, and 32 cycles (CASR) and 18, 21, 24, and 28 cycles (GAPDH) and
electrophoresed through ethidium bromide-stained agarose gels.

**FIG. 3.** RT-PCR analysis of CASR in isolated rat liver cells. RNA extracted from stellate (Ito), endothelial, Kupffer, and hepatocyte
cell populations was subjected to RT-PCR using primer set r2AF and
r4AR (Fig. 1 and Table I) generating a 1318-bp product. Aliquots of the
PCR reactions were electrophoresed through ethidium bromide-stained gels. —RT, minus reverse transcriptase control.

**FIG. 4.** Western analysis of the CASR. Rat tissue or mouse cell
extracts (10 μg of protein each) were subjected to SDS-polyacrylamide
gel electrophoresis on a 4–12% gradient gel. The blot was stained with
CASR mouse monoclonal antibody (ADD). Positive staining was demon-
strated in the liver as well as the parathyroid and kidney positive
controls but not in the NIH3T3 fibroblast negative control. The bands
were demonstrated to be specific by staining a similar blot with the
same antibody preincubated with the peptide against which it was
raised (data not shown).

**FIG. 5.** Rat hepatocytes express the CASR protein. Immunohis-
tochemistry was conducted on rat parathyroid/thyroid (A and B), kidney
(C and D), and liver (E–H) sections using a specific anti-CASR antibody
(A, C, E, and G) or the same antibody preadsorbed with CASR peptide
(B, D, F, and H). Magnification: A, B, E, and F, ×75; C, D, G, and H, ×300.
Fig. 3 shows that hepatocytes were positive for CASR mRNA expression, whereas all other cell types were negative.

**Western Blot Analysis of the Liver CASR—**Mouse cell or rat tissue extracts (10 μg of protein each) were subjected to SDS-polyacrylamide gel electrophoresis on a 4–12% gradient gel. The blot shown in Fig. 4 was stained with CASR mouse monoclonal antibody (ADD, raised against a peptide comprising CASR residues 214–236). The predominant species present in rat kidney and parathyroid are the nonglycosylated and glycosylated forms ranging from 120 to 160 kDa, with some higher molecular weight aggregates, which the rat liver lacks. A similar positive result was also obtained by Western blot analysis of an extract of isolated rat hepatocytes (data not shown). Extracts of mouse NIH3T3 fibroblasts were negative for specific CASR staining (Fig. 4), as was a blot of liver extract probed with the CASR antibody after it had been preadsorbed with the peptide against which it had been raised (data not shown).

**Immunohistochemistry of the CASR—**Strong immunostaining was observed on the chief cells of the parathyroid gland and parafollicular cells (C cells), but not the follicular cells, of the thyroid (Fig. 5A). In the kidney cortex, whereas no staining of the glomeruli was apparent, staining of the proximal and distal convoluted tubules was observed (Fig. 5C). In these controls, the pattern of staining by this particular CASR antibody was similar to that reported previously using different antibodies (44, 45). In the liver, although not all hepatocytes were found to be positive for CASR, immunostaining was observed throughout the hepatic acinus (Fig. 5E). Clear immunostaining was present in both the perivenous and periportal areas such as the hepatocytes neighboring or radiating from the central vein and those surrounding the portal triad (Fig. 5, E and G). Lack of specific staining was demonstrated in control sections treated with antibody that had been preadsorbed with CASR peptide (Fig. 5, B, D, and H).

**In Situ Hybridization of CASR mRNA—**The expression of CASR transcripts was analyzed by in situ hybridization using a biotin-labeled antisense probe. There was strong staining in endocrine cells of the parathyroid gland and the C cells of the thyroid (Fig. 6A). The specificity of the signal was demonstrated by lack of staining with the sense control probe (Fig. 6B). Specific staining was observed in hepatocytes (Fig. 6C) especially in the cells surrounding the central and portal veins (Figs. 6, C and E).

**Bile Duct Cells Do Not Express the CASR—**RNA extracted from dissected rat bile duct and a mouse bile duct cell line (BDC) was subjected to RT-PCR using primer set r2AF and r4AR (Fig. 1 and Table I). The control hepatocyte RNA was positive for the 1318-bp product, but the bile duct cell and bile duct cell line RNAs were negative (Fig. 7A). Although clear immunostaining of hepatocytes was observed, bile duct cells (Fig. 7B) were negative.

**Response of Isolated Hepatocytes to Gd^{3+}—**To test whether hepatocytes express a functional CASR, Fura-2-loaded hepatocytes were stimulated with CASR agonists. [Gd^{3+}], acts like [Ca^{2+}], on PTH release, cyclic AMP levels, and IP3 production (46) as well as [Ca^{2+}] (20) in dispersed parathyroid cells, although at a much higher potency. It was also used, rather than [Ca^{2+}], itself (which would have additionally activated Ca^{2+} channels), to monitor the isolation of the parathyroid CASR by expression cloning in Xenopus oocytes (15). In the present study we utilized [Gd^{3+}], as a well characterized ligand of the CASR. In Fura-2-loaded hepatocytes bathed in a [Ca^{2+}]-free medium, in response to 2 mM Gd^{3+}, the [Ca^{2+}], was increased and then decreased to a level about half-maximal at the end of the 200-s period and thereafter to a basal level (Fig. 8, inset). This response is not dissimilar to that observed in a subset of isolated rat pancreatic acinar cells (27), although in that case there was a longer delay before the increase in [Ca^{2+}]. In the hepatocytes there was a linear increase in [Ca^{2+}], from 1 to 3 mM Gd^{3+} (Fig. 8). A concentration of 500 μM

Fig. 6. In situ hybridization demonstrating expression of specific CASR transcripts in rat hepatocytes. Light micrographs of parathyroid/thyroid (A and B), and liver (C–F) sections with either antisense (A, C, and E) or sense (B, D, and F) CASR RNA probe are shown. Magnification: A, B, E, and F, ×75; C and D, ×300.
Gd$^{3+}$ was without effect in stimulating $[\text{Ca}^{2+}]_i$. A similar lack of $[\text{Ca}^{2+}]_i$ response to this Gd$^{3+}$ concentration was noted in studies of pancreatic acinar cells (27).

Response of Isolated Hepatocytes to Spermine—Polyamines such as spermine can stimulate $[\text{Ca}^{2+}]_i$ in HEK293 cells expressing the CASR (16), and it is suggested that tissues such as brain and intestine could use the CASR as a target for spermine and other endogenous polycations. Increased extracellular concentrations of spermine (1.25–10 mM) caused increased $[\text{Ca}^{2+}]_i$ mobilization in a dose-responsive manner in Fura-2/AM-loaded hepatocytes (Fig. 9). The effect plateaued at the 10 mM concentration with the $[\text{Ca}^{2+}]_i$ response to 20 mM spermine being no different (Fig. 9).

Effect of Prior Emptying of IP$_3$-sensitive Ca$^{2+}$ Pools on $[\text{Ca}^{2+}]_i$ Mobilization by Spermine—When Fura-2-loaded hepatocytes, bathed in a Ca$^{2+}$-free medium, had their IP$_3$-sensitive Ca$^{2+}$ pools depleted in two different ways, there was no $[\text{Ca}^{2+}]_i$ transient in response to a subsequent challenge with 2.5 mM spermine (Fig. 10). This was the case whether the Ca$^{2+}$ pool was depleted by phenylephrine, an $\alpha_1$-receptor agonist (Fig. 10, left panel), or by thapsigargin (Fig. 10, right panel). This demonstrated that the source of the $[\text{Ca}^{2+}]_i$ mobilized by CASR ligands was localized in the IP$_3$-sensitive Ca$^{2+}$ pools of the endoplasmic reticulum.

Calcimimetic NPS R-467 Enhances the $[\text{Ca}^{2+}]_i$ Response to Spermine in Hepatocytes—Phenylalkylamine compounds, NPS R-467 and NPS S-467, act as specific allosteric activators of the CASR (21). They do not activate other G protein-coupled receptors, including the related metabotropic glutamate receptors when tested at the same concentrations. The phenylalkylamine compounds act in a stereoselective manner with the $R$ enantiomer being at least 10-fold more potent than the $S$ enantiomer (21). In Fura-2-loaded hepatocytes bathed in a Ca$^{2+}$-free medium, addition of 1.25 mM spermine plus 10 $\mu$M NPS S-467 caused no greater stimulation of $[\text{Ca}^{2+}]_i$ than addition of 1.25 mM spermine alone (Fig. 11). However, addition of 1.25 mM spermine plus 10 $\mu$M NPS R-467 caused a quadrupling of the $[\text{Ca}^{2+}]_i$ response (Fig. 11). This provided good evidence that the hepatocyte polyvalent cation sensing receptor is, indeed, the CASR. It can be noted that the effect of these agents is complex in that both compounds appear to induce a delay in the response to spermine compared with the control. The underlying mechanism for this is unknown.

Spermine Enhances Bile Flow Recovery in Isolated Perfused Liver—Bile flow remained constant throughout the 60-min experimental period when normal $[\text{Ca}^{2+}]_i$ was present in the perfusate. Bile flow rapidly stopped, however, when the liver was perfused in Ca$^{2+}$-free medium despite the continuous presence of taurocholic acid to ensure normal bile acid-dependent...
bile flow. Normalization of $[\text{Ca}^{2+}]_o$ rapidly restored bile flow
with, however, a significantly more rapid bile flow recovery in
the presence of the CASR agonist spermine (Fig. 12). This effect
was dose-related, being significantly greater at 2.5 mM relative
to 1.25 mM spermine (Fig. 12, inset). The effectiveness of sper-
mine in the presence of normal $[\text{Ca}^{2+}]_o$ may relate to cooperat-
ivity between the two ions for activation of the CASR as noted
previously (16).

**CASR Agonists $\text{Ca}^{2+}$ and $\text{Gd}^{3+}$ Stimulate and NPS R-467 Selectively Enhances Bile Flow in Isolated Perfused Liver**—To
further assess the effect of CASR agonists on bile flow, isolated
livers were perfused with a 0.5 mM $\text{Ca}^{2+}$-containing medium.
For the base-line condition, the perfusate was changed at 20
min for one containing 0.5 mM $\text{Ca}^{2+}$ again. Note that this
caused a transient increase in bile flow because of the re-
equilibration of the taurocholic acid (Fig. 13). Changing to a
medium containing 1.25 mM $\text{Ca}^{2+}$ (Fig. 13A) or 0.5 mM $\text{Ca}^{2+}$ +
100 $\mu\text{M} \text{Gd}^{3+}$ (Fig. 13B) provoked a significantly increased bile
flow relative to base line. Maintaining the perfusate at 0.5 mM
$\text{Ca}^{2+}$ but adding 1 $\mu\text{M} \text{NPS R-467}$ caused a marked stimulation
of bile flow (Fig. 13C), whereas addition of 1 $\mu\text{M} \text{NPS S-467}
produced an effect little different from base line (Fig. 13D).
This provides strong evidence that activation of the CASR is
responsible for the increased bile flow.

**DISCUSSION**

In this study we have demonstrated that liver cells do ex-
press the CASR initially cloned and characterized from the
parathyroid (15) and kidney (42). This was shown by semi-
quantitative RT-PCR of total liver RNA in which low levels of
CASR PCR product were observed in the whole tissue and from
isolated hepatocytes in which there was an enrichment of the
product. Isolated nonparenchymal stellate, sinusoidal endo-
thelial, and Kupffer cells were negative for CASR mRNA. In the
case of the Kupffer cell where PTH metabolism takes place,

**Fig. 9.** Extracellular spermine increases cytosolic $\text{Ca}^{2+}$ in
hepatocytes. Rat hepatocytes loaded with Fura-2/AM were bathed in
a $[\text{Ca}^{2+}]_o$-free medium, the medium was replaced with one containing
increasing concentrations of spermine, and $[\text{Ca}^{2+}]_i$ mobilization
measured by microfluorescence. The inset shows a typical response to 2.5 mM
spermine.

**Fig. 10.** Prior emptying of IP$_3$-sensi-
tive $\text{Ca}^{2+}$ pools abrogates $[\text{Ca}^{2+}]_i$ mo-
bilization by spermine. Rat hepatocytes loaded with Fura-2/AM and bathed
in a $[\text{Ca}^{2+}]_o$-free medium and IP$_3$-sensi-
tive $[\text{Ca}^{2+}]_i$ pools were depleted by either
addition of phenylephrine (left panel) or thapsigargin (right panel) after which the
bathing solution was changed to one con-
taining 2.5 mM spermine. $[\text{Ca}^{2+}]_i$ mobiliza-
tion was monitored by microfluorescence.

**Fig. 11.** Calcimimetic NPS R-467 en-
hances the $[\text{Ca}^{2+}]_i$ response to sperm-
ine in hepatocytes. Rat hepatocytes
loaded with Fura-2/AM were bathed in a
$[\text{Ca}^{2+}]_o$-free medium. The medium was
changed to one containing 1.25 mM spermine either without or with 10 $\mu\text{M}
NPS S-467, or with 10 $\mu\text{M} \text{NPS R-467}$, and
$[\text{Ca}^{2+}]_i$ mobilization was monitored by
microfluorescence.
this result would be consistent with the lack of modulation of PTH metabolism by Ca$^{2+}$. In our study virtually the entire liver CASR mRNA protein coding region was amplified and confirmed as such by nucleotide sequencing. By Western blot analysis, using a well characterized anti-CASR monoclonal antibody, the presence of CASR protein was demonstrated in whole liver extract as well as in isolated hepatocytes. Throughout the hepatic acinus, some hepatocytes, but not all, stained positively for the CASR. Specific immunostaining was present in both the perivenous and perportal areas such as the hepatocytes neighboring or radiating from the control vein and those surrounding the portal triad. Likewise, by in situ hybridization CASR mRNA was shown to be well expressed in cells surrounding the central and portal veins. The detection of the CASR by immunohistochemistry in only a subset of hepatocytes may relate to the hepatocyte being a polarized cell. The histological preparation may expose only one side of the cell, the sinusoidal membrane (the side where the blood flows), or the canalicular membrane (the biliary side). It is not known on which side of the cell the CASR resides, but if its expression is localized this could, in part, explain the patchy staining. Some support for this hypothesis comes from the fact that the in situ hybridization signal measuring CASR mRNA was more widespread.
The CASR has been well studied with respect to its ability to stimulate inositol phosphate turnover and Ca\textsuperscript{2+} release from intracellular stores. Here, we show in isolated rat hepatocytes loaded with Fura-2/AM that when IP\textsubscript{3}-sensitive pools had been depleted by pretreatment with either thapsigargin or phenyl-ephrine, responsiveness to the CASR ligand spermine was abrogated. The pharmacological characteristics of the hepatic CASR appear to be somewhat different from those of the CASR expressed in parathyroid cells (46), distal convoluted tubule cells (13), and heterologous systems such as Xenopus oocytes (15, 42), and mammalian cells in culture (47). Thus higher [Gd\textsuperscript{3+}]\textsubscript{o} was required to stimulate [Ca\textsuperscript{2+}]\textsubscript{i} in hepatocytes than in these other cells and systems. However, similar to what we observed in hepatocytes, high [Gd\textsuperscript{3+}]\textsubscript{i} was required to elicit [Ca\textsuperscript{2+}] increases in pancreatic acinar cells (27). This may be due to the reduced density of the hepatic (and pancreatic) CASRs in the isolated cells or indicate that the CASR is coupled less efficiently to G proteins, perhaps by having its function modified by tissue-specific regulatory proteins. On the other hand, the concentrations of spermine required to promote [Ca\textsuperscript{2+}]\textsubscript{i} responses in hepatocytes were not that dissimilar from those used by Quinn et al. (16) in their study of the responsiveness to polyamines of the transiently transfected CASR in HER293 cells, especially since their studies were conducted in 0.5 mM Ca\textsuperscript{2+}, 0.5 mM Mg\textsuperscript{2+} and the present studies with hepatocytes were done in a Ca\textsuperscript{2+}-free medium.

Phenylalkylamine compounds that are positive allosteric modulators and specifically increase the sensitivity of the CASR to its ligands have been developed (18). They decrease the plasma PTH and calcium levels in patients with primary hyperparathyroidism and in dialysis patients with secondary hyperparathyroidism (21). These compounds are stereoselective (the R enantiomer is much more active than the S enantiomer) and are therefore useful in the pharmacological identification of the CASR. In the present study the responsiveness of the hepatocyte CASR to spermine was shown to be markedly enhanced in the presence of NPS R-467, whereas NPS S-467 had no effect, providing further evidence that the Ca\textsuperscript{2+} mobilization from intracellular pools was indeed a result of activation of the CASR.

What could be the physiological significance of the hepatic CASR? There is good evidence that a variety of liver functions are modulated by Ca\textsuperscript{2+}\textsubscript{i} (and, in some cases, polyamine) concentrations. These functions include bile and lipoprotein secretion, prevention of cholestasis, resistance to toxicity and injury, regeneration and proliferation, and metabolism. In the present study, we have focused on the regulation of bile secretion. The hepatocyte is the site of bile acid synthesis and plays a key role in bile formation and secretion as well as the clearance of bile acids from the portal circulation and their transport to the canalicular lumen. Calcium may play an important role in the regulation of bile secretion. A minimum perfusate Ca\textsuperscript{2+} concentration is required for normal bile secretion from perfused rat liver (48, 49). Reduction of calcium in rat liver perfusate leads to increased paracellular permeability and a decrease of both biliary excretion and bile flow. At very low calcium levels bile flow is virtually abolished (50). A similar decrease in bile secretion was found in the hypocalcemic thyroparathyroidectomized rat (51, 52). The present study indicates that in the rat liver, bile flow is very sensitive to the prevailing extracellular Ca\textsuperscript{2+} concentration. The CASR agonist spermine enhanced the rate of restoration of bile flow in perfused liver preparations following a period of cholestasis induced by calcium deprivation. This suggested that the CASR might play an important role in bile secretion. Further evidence of this was provided by the finding that the bile secretion rate obtained at reduced calcium concentration (0.5 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}) could be stimulated by increasing the calcium concentration or perfusing another CASR agonist, Gd\textsuperscript{3+}. Importantly, addition of 1 µM NPS R-467 (but not the inactive enantiomer, NPS S-467) markedly stimulated bile flow, providing strong evidence for the involvement of the CASR.

The suggested role of the CASR in bile secretion is compatible with its localization in hepatocytes rather than bile duct itself. It is known that cells within the hepatic acinus display important functional differences according to their location, although all hepatocytes contribute to the formation of bile. However, the rate of extraction of bile acids is highest in the perportal region, suggesting that peripoal cells contribute a greater proportion of the bile acid-dependent flow, whereas perivenous hepatocytes contribute a greater proportion of the bile acid-independent flow. However, there is substantial overlap between these mechanisms (53). In our perfused liver preparation, taurocholate was present to ensure both bile acid-dependent as well as bile acid-independent bile flow. Additional studies will be required to refine the precise cellular localization of the CASR and elucidate its role in the different bile flow mechanisms.

Familial (benign) hypocalciuria hypercalcemia (FHH) is an autosomal dominant disorder caused by inactivating mutations in the CASR (see Ref. 8 for review). Law and Heath (54) reported clinical findings on 21 families with FHH. With respect to clinical correlates of hepatic expression of the CASR, they found a higher incidence of FHH-affected family members with gall stones than in the general population. Some reports (55–57) have suggested that FHH can be associated with recurrent pancreatitis. Pancreatitis is most often secondary to alcoholism or biliary tract disease and less often to other causes including hyperparathyroidism. In the exocrine pancreas, the CASR is present in acinar cells and interlobular ducts, and in the latter cells activation of the CASR stimulates fluid secretion (27). It is suggested that the CASR monitors the Ca\textsuperscript{2+} concentration in the pancreatic juice and regulates the level of Ca\textsuperscript{2+} in the lumen, normally preventing the occurrence of conditions leading to pancreatic stone formation and pancreatitis. The CASR in hepatocytes may preserve a similar function and monitor the Ca\textsuperscript{2+} concentration in the bile secreted from hepatocytes. There is the potential for involvement of altered polyvalent cation sensing in the etiology of hepatic and (associated) pancreatic disorders.

Finally, retention of biliary constituents such as bile salts during cholestasis may result in hepatic damage and apoptosis (58). It has been demonstrated in other cell models that elevated [Ca\textsuperscript{2+}]\textsubscript{i}, can act as a first messenger to prevent apoptotic cell death and that the protective effects are mediated by the CASR (59). Therefore, potent and tissue-specific allosteric modulators of the CASR may be useful in states in which apoptosis is thought to contribute to liver damage such as alcohol-induced hepatitis, drug-induced liver diseases or during organ storage before liver transplantation.

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Extracellular Calcium-sensing Receptor Is Expressed in Rat Hepatocytes: COUPLING TO INTRACELLULAR CALCIUM MOBILIZATION AND STIMULATION OF BILE FLOW
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