The balance between the concentrations of free ionized Ca\(^{2+}\) and bicarbonate in pancreatic juice is of critical importance in preventing the formation of calcium carbonate stones. How the pancreas regulates the ionic composition and the level of Ca\(^{2+}\) saturation in an alkaline environment such as the pancreatic juice is not known. Because of the tight cause-effect relationship between Ca\(^{2+}\) concentration and lithogenicity, and because hypercalcemia is proposed as an etiologic factor for several pancreatic diseases, we have investigated whether pancreatic tissues express a Ca\(^{2+}\)-sensing receptor (CaR) similar to that recently identified in parathyroid tissue. Using reverse transcriptase-polymerase chain reaction and immunofluorescence microscopy, we demonstrate the presence of a CaR-like molecule in rat pancreatic acinar cells, pancreatic ducts, and islets of Langerhans. Functional studies, in which intracellular free Ca\(^{2+}\) concentration was measured in isolated acinar cells and interlobular ducts, show that both cell types are responsive to the CaR agonist gadolinium (Gd\(^{3+}\)) and to changes in extracellular Ca\(^{2+}\) concentration. We also assessed the effects of CaR stimulation on physiological HCO\(_3\)\(^-\) secretion from ducts by making measurements of intracellular pH. Luminal Gd\(^{3+}\) is a potent stimulus for HCO\(_3\)\(^-\) secretion, being equally as effective as raising intracellular cAMP with forskolin. These results suggest that the CaR in the exocrine pancreas monitors the Ca\(^{2+}\) concentration in the pancreatic juice, and might therefore be involved in regulating the level of Ca\(^{2+}\) in the lumen, both under basal conditions and during hormonal stimulation. The failure of this mechanism might lead to pancreatic stone formation and even to pancreatitis.

Pancreatic juice in humans and other species is an alkaline secretion, containing up to 140 mM bicarbonate ions (1). The juice also contains millimolar quantities of Ca\(^{2+}\) ions, which are released from secretory granules along with pancreatic zymogens (1). The presence of these ions means that the pancreatic juice is at risk of precipitating calcium stones, which are a major cause of chronic pancreatitis (1). This is especially true when the residence time of the juice within the ductal compartment is increased, i.e., at low pancreatic secretory rates. Because the incidence of pancreatic stone formation is surprisingly low, it has been postulated that homeostatic responses must be activated to reduce lithogenic potential. A number of suggestions have been advanced for candidate mechanisms, all within the ductal system, including increased H\(^+\) and fluid secretion and a reduction in bicarbonate production (1). Moreover, thus far there is no direct experimental evidence for any of these hypotheses, and the question of how the pH and the free Ca\(^{2+}\) concentration in pancreatic juice are regulated is yet to be understood.

Recently Brown et al. (2) have identified a cell-surface, G protein-linked Ca\(^{2+}\) (polycation cation)-sensing receptor (CaR)\(^1\) which is capable of monitoring even minute changes in extracellular calcium concentration ([Ca\(^{2+}\)]\(_o\)) and responds with an increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)). The receptor is sensitive to changes in [Ca\(^{2+}\)]\(_i\), in the millimolar range, compatible with [Ca\(^{2+}\)]\(_i\), in the plasma. Intriguingly, the concentration of free ionized Ca\(^{2+}\) in human pancreatic juice under conditions of basal secretion has been estimated to be ~1 mM (1). Since hypercalcemia stimulates pancreatic secretion (3), and is associated with pathological effects including acinar and ductal cell necrosis, formation of intraductal precipitates, and clinical pancreatitis (3–6), we tested the hypothesis that a CaR-like mechanism was also expressed in the rat exocrine pancreas.

Reverse-transcriptase PCR on total rat pancreas mRNA, using intron-spanning primers, established the presence of CaR-like transcripts, and immunological localization of CaR protein employing an anti-CaR polyclonal antiserum revealed strong immunoreactivity at the luminal side of pancreatic ducts and a more diffuse, cellular and basolateral staining pattern in acinar cells. The receptor is also strongly expressed in clusters of cells representing the islets of Langerhans. Functional studies in isolated pancreatic acinar cells, and in microperfused interlobular ducts, using the Ca\(^{2+}\)-sensitive fluorescent dye Fura-2 showed that the CaR agonists [Ca\(^{2+}\)]\(_o\) and Gd\(^{3+}\) raised [Ca\(^{2+}\)]\(_i\) in both acini and ducts. Finally, we tested the hypothesis that the CaR might play a role in regulating ductal HCO\(_3\)\(^-\) secretion. Luminal microperfusion of Gd\(^{3+}\) resulted in enhanced ductal HCO\(_3\)\(^-\) secretion, comparable to that evoked by the secretagogue forskolin (7).

Our findings indicate that the CaR in the pancreas plays a significant role in the regulation of pancreatic juice secretion, in particular by constantly monitoring the level of free Ca\(^{2+}\) in secreted fluid within the acinar and duct lumen. Alterations of

\(^{1}\)The abbreviations used are: CaR, Ca\(^{2+}\)-sensing receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; CCK, cholecystokinin; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; BCECF, 2',7'-bis(2-carboxyethyl)-5-carboxyfluorescein.
receptor function could help explain how ion sensing by the exocrine pancreas can go awry, resulting in pancreatic stone formation and acute pancreatitis.

**EXPERIMENTAL PROCEDURES**

Reverse Transcriptase-PCR (RT-PCR)—Male Sprague-Dawley rats weighing between 100 and 300 g were killed by cervical dislocation. The pancreas was removed, frozen immediately in liquid N\_2, and stored at ~80 °C until use. Total RNA was extracted using the guanidinium thiocyanate/acid phenol method (8). First strand DNA was synthesized from 1 to 2 μg of total RNA using SuperScript Reverse Transcriptase (Life Technologies) according to the manufacturer’s instructions and the resultant first-strand DNA was then used for PCR amplification. In order to perform “hot start” PCR, the Taq DNA polymerase was added during the initial 3-min denaturation, followed by 35 cycles of amplification (1 min denaturation at 92 °C, 30 s annealing at 47 °C, and 1 min extension at 72 °C), with final extension for 10 min at 72 °C. The 383 base pairs expected PCR product was visualized with ethidium bromide after electrophoretic separation on a 1% agarose gel, and the specificity of the PCR reaction was assessed by high stringency Southern blotting using a 32P-labeled 3.2-kilobase Xho-I fragment corresponding to the coding region of the rat kidney CaR (9, 10), as described elsewhere (9). To avoid genomic DNA amplification, the primer sequences were based on the known rat CaR sequence (10) and designed to span one intron: forward primer, 5′-ACCTTATGCCATCCTGGTA3′; reverse primer, 5′-GGGCCAAGAATCTCAAGGTG-3′. As negative and positive controls the DNA template was replaced with an equivalent amount of total RNA reverse-transcribed from rat kidney, respectively.

**Immunoreactivity of CaR Protein in Rat Pancreas—**CaR polyclonal antibody raised against a 20-amino acid sequence in the predicted hydrophilic amino-terminal region of the bovine CaR (11) were generously provided by Drs. S. C. Hebert (Vanderbilt University, Nashville, TN) and by Dr. E. M. Brown (Harvard Medical School, Boston, MA). Prior to use, the antibodies were purified using an affinity column based on the known rat CaR sequence (11) and were designed to span one intron: forward primer, 5′-ACCTTATGCCATCCTGGTA3′; reverse primer, 5′-GGGCCAAGAATCTCAAGGTG-3′. As negative and positive controls the DNA template was replaced with an equivalent amount of total RNA reverse-transcribed from rat kidney, respectively.

**Isolation and Culture of Interlobular Ducts—**Interlobular ducts were prepared as described previously (15). Animals were killed by cervical dislocation and isolated by digesting the tissue with collagenase. Ducts were collected on Filterpaper and snap-frozen in liquid N\_2. After being stored at −80 °C, the ducts were thawed, washed with HEPES-buffered saline containing either 5 mM of Ca\(^{2+}\), and pH 7. The ducts were then transferred to a perfusion chamber for further 30 min. The digested tissue was washed with Dulbecco’s modified Eagle’s medium and resuspended in Dulbecco’s modified Eagle’s medium containing 0.2 mg ml\(^{-1}\) soybean trypsin inhibitor, and 0.2% (w/v) bovine serum albumin. Interlobular ducts (diameter 100–130 μm) were microdissected from samples of this tissue suspension under a dissecting microscope using sharpened needles. The ducts were then placed on polycarbonate membrane filters (Cyclopore) floating on McCoy’s 5A tissue culture medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mg ml\(^{-1}\) soybean trypsin inhibitor, and 0.2% (w/v) bovine serum albumin. The ducts were transferred to a perfusion chamber (volume 200 μl) mounted on the stage of a Nikon Diaphot inverted microscope for simultaneous luminal microperfusion and measurement of [Ca\(^{2+}\)]. The duct was gently aspirated into a holding pipette (tip diameter 50–80 μm), and a perfusion pipette (tip diameter 10 μm) was then advanced into the duct lumen for luminal perfusion. The end of the perfusion pipette was then lowered to the bottom of the duct lumen and the position of the bottom of the duct lumen was recorded as described previously (15). The Ca\(^{2+}\) influx was measured by dual-excitation wavelength microfluorometry as described previously (15, 16). An optical diaphragm in the emitted light path limited excitation to a small region of the duct lumen (10–20 μm in diameter). Records of [Ca\(^{2+}\)] were displayed as the uncalibrated Fura-2 fluorescence ratio, while records of pH, were calibrated using nigericin/high K\(^{+}\) (15).

**Loading of Pancreatic Acinar Cells with Fura-2-AM and Recording of [Ca\(^{2+}\)]—**Acinar cells were loaded with 4 μM Fura-2-AM (Molecular Probes) for 40 min at room temperature, and then transferred to a perfusion chamber as described previously (14). Once cells had adhered to the chamber base they were continuously superfused with HEPES-buffered saline from a gravity-fed perfusion system at a rate of 2 ml min\(^{-1}\). The intensity of Fura-2 fluorescence in a field of up to 30 acinar cells was imaged using a Nikon Diaphot microscope and a low-scan CCD camera (Digital Pixel Ltd, Brighton, UK) as described previously in detail (14). Background-subtracted 340/380 images were calculated off-line and used to generate records of the 340/380 ratio in each of the individual acinar cells within the microscopy field.

The composition of the physiological saline used in acinar cell imaging experiments was similar to that in the isolation procedure, but omitting amino acids, glutamine, trypsin inhibitor, and bovine serum albumin. In addition, SO\(_4\)\(^{2-}\) and HPO\(_4\)\(^{2-}\) ions were replaced with Cl\(^-\) in experiments employing gadolinium and high [Ca\(^{2+}\)] in order to prevent precipitation of insoluble gadolinium or calcium salts. In most experiments Ca\(^{2+}\) was reduced to 50 μM in order to prevent activation of the CaR. Preliminary experiments established that 0.1 mM CaCl\(_2\) was the minimum necessary to maintain [Ca\(^{2+}\)]\(_i\), oscillations evoked by the physiological secretory agonist cholecystokinin (CCK). As an index of viability only cells which responded to 50 pM CCK were utilized. All solutions and experimental media were equilibrated with 100% O\(_2\) and all experiments were carried out at room temperature.
**RESULTS**

**Expression of CaR-related Transcripts in Rat Pancreas**

Reverse transcriptase PCR using specific, intron-spanning primers was followed by high stringency Southern analysis. The expected 383-base pair size product was amplified from rat pancreas (P), rat kidney (positive control, +) but not when H₂O replaced DNA samples in the PCR reaction (negative control, −).

100% O₂. When the perfusate contained 0.5, 5, or 8 mM Ca²⁺, NaCl concentration was reduced appropriately to maintain isomolarity. All HEPES-buffered solutions were adjusted to pH 7.4 at 37 °C. For experiments assessing ductal HCO₃⁻ secretion, the bath was perfused with a medium containing 25 mM HCO₃⁻ (replacing Cl⁻) and equilibrated with 95% O₂, 5% CO₂ to give a pH of 7.4, while the lumen was perfused with the HEPES-buffered perfusion fluid described above.

**Immunofluorescence and Immunohistochemistry**

In order to identify which cells in the rat pancreas expressed the CaR, we carried out immunolocalization with an affinity-purified anti-CaR polyclonal antibody raised against the bovine parathyroid CaR (11). Light microscope images (Fig. 2) revealed that specific CaR immunoreactivity, visualized using peroxidase staining, was present in ducts, acini, and also in cells within the islets of Langerhans. Similar results were also seen when a fluorophore-coupled, rather than horseradish peroxidase-conjugated, secondary antibody was used (data not shown).

The intensity of the staining observed in acinar cells was often difficult, we went on to define the subcellular localization of the CaR in the same isolated interlobular duct preparation used for functional studies. As shown in Fig. 2F, a strong immunofluorescence signal was observed inside the ducts, representing the luminal membrane of the duct cells.

**Functional Evidence for a CaR in Exocrine Pancreas**

Response of Isolated Acini to Gd³⁺—In order to test whether acinar cells express a functional CaR, we stimulated fura-2-loaded acinar cells with CaR agonists. Initially we tested 1 mM Gd³⁺, which produced changes in [Ca²⁺]ᵢ in 53% of cells (Fig. 3), when applied either before or after stimulation with 50 pM CCK. Treatment with 1 mM Gd³⁺ after CCK stimulation triggered responses in approximately half (53%) of the acinar cells tested, with a range of response profiles being observed (Fig. 3).

A rapid and irreversible increase in [Ca²⁺]ᵢ was evoked in 6% of cells (Fig. 3A), a large transient increase in [Ca²⁺]ᵢ in 9% of cells (Fig. 3B), oscillations in [Ca²⁺]ᵢ in 31% of cells (Fig. 3C), and a slow rise in [Ca²⁺]ᵢ in 7% of cells (Fig. 3D). Finally, no change in [Ca²⁺]ᵢ was detected in 47% of cells (Fig. 3E; all data derived from a total of 93 cells from 4 rats).

Treatment with 1 mM Gd³⁺ prior to CCK stimulation caused a rapid and transient increase in [Ca²⁺]ᵢ in 18% of cells, oscillations in [Ca²⁺]ᵢ in 31%, a slow rise in [Ca²⁺]ᵢ in 25%, and no change [Ca²⁺]ᵢ in 25% of cells (all values n = 83 cells from 3 rats). The smaller percentage of cells showing no response to Gd³⁺, as compared with cells stimulated with Gd³⁺ after CCK treatment, suggests that cells were slightly more sensitive to Gd³⁺ when treated prior to CCK stimulation.

We also treated cells with a lower concentration of 600 μM Gd³⁺, typically the maximum concentration which has been used in heterologous expression systems (e.g. Xenopus laevis oocytes and human embryonic kidney cells (see Ref. 2 for review). Somewhat to our surprise, this failed to produce any change in [Ca²⁺]ᵢ.
Response of Isolated Acini to Elevated \([\text{Ca}^{2+}]_o\) — We went on to test acinar cells with the physiological agonist of the CaR by examining the effects of raising \([\text{Ca}^{2+}]_o\) from 0.1 to 8 mM (Fig. 4). This produced a variety of changes in \([\text{Ca}^{2+}]_o\). However, the pattern of changes was noticeably different from the profile of responses observed with Gd\(^{3+}\) (Fig. 3). Only 20% of cells produced oscillations in \([\text{Ca}^{2+}]_o\), and the oscillations were superimposed over a slowly rising baseline (Fig. 4A), which did not occur with Gd\(^{3+}\) (compare Fig. 3). In 16% of cells a transient increase in \([\text{Ca}^{2+}]_o\) was observed (Fig. 4B). However, the kinetics of this change in \([\text{Ca}^{2+}]_o\) were different from the equivalent Gd\(^{3+}\)-evoked response (Fig. 3B) as a much slower increase was observed with high \([\text{Ca}^{2+}]_o\). A slow and smaller increase in \([\text{Ca}^{2+}]_o\), which continued throughout the exposure to 8 mM \([\text{Ca}^{2+}]_o\), was observed in 17% of cells (Fig. 4C) while 47% of cells failed to produce a response (Fig. 4D; \(n = 140\) cells, 5 rats). Overall, it was clear that fewer cells showed typical receptor-mediated responses when stimulated with elevated \([\text{Ca}^{2+}]_o\) than when stimulated with Gd\(^{3+}\). In fact the predominant response seemed to be a slow increase in baseline \([\text{Ca}^{2+}]_o\), seen in around 50% of cells, which is suggestive of increased Ca\(^{2+}\) entry.

Finally, we tested the polycationic antibiotic neomycin (500 \(\mu\text{M}\) and 1 mM), another known CaR agonist (2). In our hands neomycin failed to produce any change in \([\text{Ca}^{2+}]_o\) in all acinar cells tested (\(n = 48\) cells from two rats for 500 \(\mu\text{M}\) neomycin, 156 cells from six rats for 1 mM) (not shown).

Response of Isolated Interlobular Ducts to Gd\(^{3+}\) — We examined the effects of Gd\(^{3+}\) on interlobular ducts with the same experimental protocol used to test for CaR expression in studies on heterologous expression systems (2). In this protocol Gd\(^{3+}\) is applied in the absence of all other divalent cations (i.e. in the absence of extracellular Ca\(^{2+}\) and Mg\(^{2+}\)). We first performed preliminary experiments to ensure the viability of the isolated ducts in the absence of divalent cations. This was achieved by perfusing both bath and lumen with Ca\(^{2+}\)- and Mg\(^{2+}\)-free solution for at least 10 min and then testing duct viability by the response to bath application of 10 \(\mu\text{M}\) acetylcholine (ACh, not shown). Having established that the ducts remained viable in the absence of divalent cations, we determined the functional localization of the CaR (apical versus basolateral). Ducts were challenged with 600 \(\mu\text{M}\) Gd\(^{3+}\) using three different approaches: simultaneous luminal and bath perfusion, luminal perfusion only, or basolateral perfusion only. In all experiments we could only detect changes in \([\text{Ca}^{2+}]_o\) when Gd\(^{3+}\) was applied luminally (\(n = 31\) ducts from four rats).

Bath perfusion with Gd\(^{3+}\) failed to produce an increase in \([\text{Ca}^{2+}]_o\) (\(n = 8\) ducts from four rats).

Under these conditions, all of the ducts tested responded to luminal Gd\(^{3+}\) with an increase in \([\text{Ca}^{2+}]_o\). Fig. 5 shows a representative response, in which \([\text{Ca}^{2+}]_o\), slowly increased when Gd\(^{3+}\) was added to the luminal solution. Little or no recovery of \([\text{Ca}^{2+}]_o\), was observed on removal of Gd\(^{3+}\) in this experiment. This was typical of 23 out of 31 ducts. Recovery to baseline after Gd\(^{3+}\) stimulation was observed in only 26% of responses (\(n = 8\) ducts from four rats; not shown).

We went on to challenge ducts with high concentrations of \([\text{Ca}^{2+}]_o\) (5 or 8 mM). In initial experiments a slow increase in \([\text{Ca}^{2+}]_o\), was seen on raising \([\text{Ca}^{2+}]_o\), in either the bath or the luminal perfusate (not shown). The effects of bath \([\text{Ca}^{2+}]_o\), on \([\text{Ca}^{2+}]_o\), have been previously reported (16), and are thought to be due to Ca\(^{2+}\) entry, presumably across the basolateral membrane. We found that, in order to obtain reproducible responses to changes in luminal \([\text{Ca}^{2+}]_o\), it was necessary to ensure that intracellular Ca\(^{2+}\) stores remained loaded with Ca\(^{2+}\) during the period of low \([\text{Ca}^{2+}]_o\), perfusion. This was achieved by perfusing the bath with 1 mM \([\text{Ca}^{2+}]_o\), to maintain loading of Ca\(^{2+}\) into the intracellular stores, while perfusing the lumen with a lower concentration of \([\text{Ca}^{2+}]_o\), (0.5 mM). Under these conditions (Fig. 6), increasing luminal \([\text{Ca}^{2+}]_o\), to 8 mM evoked an increase in \([\text{Ca}^{2+}]_o\), which was qualitatively and quantitatively similar to that stimulated by addition of Gd\(^{3+}\) (Fig. 6) (\(n = 9\) ducts from two rats).

Finally, we tested whether the CaR in the ducts could be activated by neomycin (500 \(\mu\text{M}\) and 1 mM, not shown). We did not observe a change in \([\text{Ca}^{2+}]_o\), in any of the ducts tested (\(n = 9\) ducts from two rats), although the same ducts remained responsive to luminal Gd\(^{3+}\) (600 \(\mu\text{M}, n = 4\) ducts from two rats) and/or bath ACh (10 \(\mu\text{M}, n = 9\) ducts from two rats). These data agree with those obtained in acinar cells and indicate that neomycin does not act as an agonist of the CaR in rat pancreas.

Stimulation of Ductal Secretion by Luminal Gd\(^{3+}\) — In order to test the hypothesis that the luminal CaR can stimulate fluid secretion in the ductal system, we measured the rate of cellular
HCO₃⁻ efflux in microperfused ducts. Pancreatic ducts secrete a Na⁺- and HCO₃⁻-rich fluid, so that the rate of ductal HCO₃⁻ secretion also gives an index of the rate of fluid secretion (7, 15, 17). We have previously shown that stimulation of isolated ducts with the cAMP-elevating hormone secretin causes little change in intracellular pH (pHᵢ) because the increased rate of luminal (secretory) HCO₃⁻ efflux is balanced by increased basolateral HCO₃⁻ uptake (15). This basolateral HCO₃⁻ uptake is mediated by the basolaterally located Na⁺-HCO₃⁻ co-transporter and Na⁺-H⁺ exchanger (15). Consequently, inhibition of these basolateral transporters with a combination of H₂DIDS and amiloride reveals a marked intracellular acidification which can be attributed to luminal HCO₃⁻ efflux (7, 15). Fig. 7A shows typical records of such cellular acidification (HCO₃⁻ secretion) in control ducts and in ducts treated with forskolin or with luminal Gd³⁺. The averaged data in Fig. 7B show that forskolin, which raises intracellular cAMP by stimulating adenylate cyclase, and evokes secretion of fluid and HCO₃⁻ (15), caused an approximate doubling of the cellular acidification rate (HCO₃⁻ secretory rate). This is similar to the increase in HCO₃⁻ secretory rate that we have previously reported for stimulation with secretin (15). Perfusion of the duct lumen with 600 μM Gd³⁺ increased the acidification rate to approximately the same extent as forskolin. This demonstrates that activation of luminal CaRs can indeed activate ductal HCO₃⁻ (and hence fluid) secretion.

**DISCUSSION**

In this study we have investigated the hypothesis that regulation of pancreatic juice ionized Ca²⁺ concentration involves an extracellular CaR within the pancreas. The first molecular evidence for the presence of such a receptor came from RT-PCR. As shown in Fig. 1, high-stringency Southern analysis using a CaR probe confirmed the specificity of the PCR amplification product. Immunofluorescence and confocal microscopy subsequently revealed expression of CaR protein in acinar, duct, and islet cells, in each case with a different and characteristic staining pattern at the subcellular level: basolateral/intracellular in the acini, luminal in the ducts, and at the periphery of cells composing the islets of Langerhans.

**FIG. 4. Effect of elevated extracellular Ca²⁺ on intracellular free calcium concentration in isolated pancreatic acinar cells.** Isolated Fura-2-loaded acinar cells were superfused with sulfate- and phosphate-free medium containing 0.1 mM Ca²⁺ and exposed to 50 μM CCK and then, following a recovery period, to medium containing 8 mM Ca²⁺. The four panels A-D show the range of different types of [Ca²⁺]ᵢ responses to Gd³⁺ observed, as follows: A, oscillations; B, transient increase; C, slow increase in baseline; and D, no response. The percentage of cells showing each type of response is indicated in the pie chart. A total of 140 individual cells was analyzed in five separate experiments on cells derived from different animals.

**FIG. 5. Effect of Gd³⁺ on intracellular free calcium concentration in a microperfused, Fura-2-loaded, interlobular pancreatic duct.** Initially both the bath and the duct lumen were perfused with sulfate- and phosphate-free medium containing no added Ca²⁺ or Mg²⁺. Gadolinium (600 μM) was included in the luminal and the bath perfusate as indicated by the bars. The trace is representative of 8 ducts for bath exposure to Gd³⁺, and 23 ducts for luminal exposure to Gd³⁺.
Acinar cells exhibit diffuse, punctate staining, indicating that the receptor is not homogeneously distributed at the cell surface (see Fig. 2). Staining was predominantly observed at the basolateral side of acinar cells, consistent with the presence of the CaR on the basolateral membrane. This agrees well with the functional studies showing agonist effects of Gd$^{3+}$ and Ca$^{2+}$. In addition, however, significant intracellular and more apical staining was also detected. There are two possible explanations for this. First, pancreatic acinar cells have a high rate of luminal exocytosis (zymogen secretion). Endocytosis accompanies this exocytosis as part of the normal process of membrane recycling (18). It is therefore conceivable that, if the CaR is present on the acinar cell apical membrane, immunolocalization would reveal primarily intracellular staining as the receptor is recycled.

Alternatively, the intracellular staining may indicate a true intracellular localization of the receptor. This might reflect a high rate of receptor biosynthesis, or extensive post-translational modification occurring prior to receptor insertion into the plasma membrane, or even some functional role. An intracellular punctate distribution pattern is not unusual for the CaR, and has previously been observed in chief cells of the bovine parathyroid gland (19). Interestingly, in these cells the receptor is localized within caveolin-rich intracellular membrane domains where it has been suggested to regulate parathyroid hormone secretion via phosphorylation/dephosphorylation (20).

It is now well established that the CaR stimulates Ca$^{2+}$ release from intracellular stores via a phosphoinositide pathway (2, 10). When we exposed pancreatic acinar cells to 1 mM Gd$^{3+}$, changes in [Ca$^{2+}$] were evoked in between 50% (CCK prior to stimulation with Gd$^{3+}$) and 75% (Gd$^{3+}$ prior to CCK administration) of all acinar cells, with oscillations in [Ca$^{2+}$] being observed in about a third of the cells. The lack of any response to Gd$^{3+}$ or high [Ca$^{2+}$], in between 50 and 25%, respectively, of all cells could be ascribed to the possibility that the enzymatic digestion procedure employed to isolate single cells damages some of the cell-surface receptors. This is a particular concern with the CaR, because it possesses a very large extracellular domain (2, 10). Indeed, damage of this kind might explain why Gd$^{3+}$ was a more effective stimulus than high [Ca$^{2+}$], or neomycin, since Hammerland et al. (21) showed by mutagenesis that removal of the extracellular domain of the CaR abolishes the response to Ca$^{2+}$ and neomycin but partly retains the response to Gd$^{3+}$. In addition, previous observations in human pancreatic insulinoma cells are consistent with the presence of a Ca$^{2+}$-sensing mechanism activated by Gd$^{3+}$ but not by neomycin (22).

Overall, the nature and kinetics of the [Ca$^{2+}$], responses
observed with Gd$^{3+}$ are characteristic of responses to a G-protein/phosphoinositide-linked agonist in acinar cells, and notably resemble responses to submaximal doses of CCK (23). Under these circumstances some cells, representing those least sensitive to CCK, produce no response, while a small proportion of cells, representing the cells with the highest CCK sensitivity, produce a large sustained or transient increase in $[\text{Ca}^{2+}]_o$. The majority of cells, possessing intermediate CCK sensitivity, produce oscillations in $[\text{Ca}^{2+}]_o$. Although the coupling of the CaR to the phosphoinositide/Ca$^{2+}$ pathway is well established, to our knowledge this is the first demonstration that activation of the CaR evokes $[\text{Ca}^{2+}]_o$ oscillations.

Treatment with Gd$^{3+}$ was somewhat more effective in raising $[\text{Ca}^{2+}]_o$ when cells had not previously been stimulated with CCK. It is possible that treatment with CCK prior to Gd$^{3+}$ may partially deplete the intracellular Ca$^{2+}$ stores, especially since $[\text{Ca}^{2+}]_o$ in these experiments was only 0.1 mM, which might reduce store re-filling. This is consistent with the observation that the stimulatory effect of hypercalcemia on feline pancreatic secretion could be prevented when a large dose of CCK was infused prior to induction of hypercalcemia (3).

Consistent with previously reported observations (2, 10), elevated $[\text{Ca}^{2+}]_o$ appeared less effective than Gd$^{3+}$ as an agonist of the CaR in pancreatic acinar cells (see “Results”). Similar results were also obtained in isolated ducts, where Gd$^{3+}$-induced responses were more consistent than those evoked by increased $[\text{Ca}^{2+}]_o$. Interpretation of experiments with high $[\text{Ca}^{2+}]_o$ is complicated by the fact that Ca$^{2+}$ will leak into the cell via Ca$^{2+}$ channels. It is unlikely, however, that this would produce rapid spike-like increases in $[\text{Ca}^{2+}]_o$, or $[\text{Ca}^{2+}]_o$ oscillations. The most likely effect of increased Ca$^{2+}$ entry would be a much slower rise in $[\text{Ca}^{2+}]_o$, as was indeed observed in around 40% of acinar cells and in three-quarters of the isolated ducts. This kind of very gradual rise in $[\text{Ca}^{2+}]_o$, was never observed with the cell-impermeant CaR agonist Gd$^{3+}$.

The characteristics of the CaR in the pancreas appeared slightly different from those previously described in heterologous expression systems (2, 10). First, we found that it was necessary to use 1 mM Gd$^{3+}$ to elicit $[\text{Ca}^{2+}]_o$ responses in acinar cells, rather than the 600 mM used in most heterologous expression systems. Second, the response to 8 mM Ca$^{2+}$ was also rather poor compared with that reported in cells overexpressing the CaR (2, 10). Finally, neomycin failed to produce any change in $[\text{Ca}^{2+}]_o$, in either acinar or duct cells. It is not entirely clear whether these data imply that the receptor in pancreatic acinar and duct cells is distinct from the CaR isolated from parathyroid or kidney cells, since damage to the extracellular domain of the receptor during enzymatic digestion (see above) could perhaps explain the reduced agonist sensitivity. In addition, the failure of neomycin to increase $[\text{Ca}^{2+}]_o$, in acinar and duct cells could reflect the fact that millimolar levels of neomycin are known to inhibit phospholipase C activity and therefore inositol trisphosphate production (24, 25). Another possibility would be that the pharmacology of the CaR might be influenced by other regulatory proteins with tissue-specific distribution. However, the hypothesis of a different CaR subtype with a reduced affinity for Ca$^{2+}$ and polyvalent cations in the pancreas cannot be entirely ruled out, particularly given (i) the recent identification of a novel CaR splice variant in keratinocytes (26) and (ii) the possible existence of multiple CaR genes (27). Although our data provide the first functional evidence for the existence of a CaR in pancreatic acinar cells, data preceding the cloning of the CaR in 1993 are consistent with our findings. For instance, elevating perfusate $[\text{Ca}^{2+}]_o$ to 5 mM greatly potentiates amylase secretion from the cat pancreas (28), while Ma-ruyama (29) showed in whole cell patch-clamp experiments that extremely high concentrations (>50 mM) of divalent cations (Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, Ni$^{2+}$, and Mg$^{2+}$) activated Ca$^{2+}$-dependent currents in pancreatic acinar cells by triggering intracellular Ca$^{2+}$ release. These latter results were ascribed by the author to the activation of a nonspecific cell surface receptor or receptor-effector complex via the indirect action of cations on membrane surface charge (29). In retrospect, the data are quite consistent with the presence of the CaR, although a nonspecific effect on membrane charge cannot be ruled out.

The presence of the CaR in pancreatic acinar cells may have physiological implications, as it suggests that increased $[\text{Ca}^{2+}]_o$ may be able to stimulate exocrine secretion in the absence of hormonal or neuronal stimulation. The exact concentration of Ca$^{2+}$ required to stimulate secretion in vivo would be very difficult to deduce from this study. However, given that CaR(s) are sensitive to values of $[\text{Ca}^{2+}]_o$, in the physiological range, it is tempting to speculate that stimulation of the CaR might play a role in producing the basal in vivo pancreatic secretion which is observed in a number of species (3). In addition, several lines of evidence indicate that hypercalcemia induces acinar cell damage and eventually pancreatitis (6). The largest effects are obtained with Ca$^{2+}$ concentrations ranging between 0.6 and 5 mM (5), consistent with the range of activation of the CaR (2). Our data suggest the intriguing hypothesis that these effects might be mediated by CaR present at the basolateral surface of acinar cells.

A further possible functional role for the CaR in acinar cells might be in controlling cell proliferation. Proliferation-associated pathways are known to be present in pancreatic acinar cells and are thought to be activated by CCK (30, 31). Given the similar $[\text{Ca}^{2+}]_o$, responses evoked by CCK and Gd$^{3+}$, it may be that the CaR in acinar cells helps to transduce a proliferative stimulus driven by extracellular Ca$^{2+}$.

**CaR in Exocrine Duct Cells**—In contrast to acinar cells, duct cells exhibit a strong CaR immunostaining pattern along the apical surface delimiting the lumen of the ducts, suggesting that the receptor is localized to the luminal membrane. This staining pattern was observed both in tissue sections and in the isolated duct preparation used for the functional studies. It was also confirmed by the functional studies, which showed that Gd$^{3+}$ only elevated duct $[\text{Ca}^{2+}]_o$, when included in the luminal perfusate.

The effect of luminal Gd$^{3+}$ on $[\text{Ca}^{2+}]_o$, in ducts was very consistent, namely a gradual increase. No oscillations were observed, but this is not surprising given that oscillations in $[\text{Ca}^{2+}]_o$, have never been reported in agonist-stimulated duct cells. Although the gradual increase in $[\text{Ca}^{2+}]_o$, could conceivably also be attributed to an inhibition of Ca$^{2+}$ extrusion by Gd$^{3+}$, this seems unlikely given that Gd$^{3+}$ only raised $[\text{Ca}^{2+}]_o$, when applied to the luminal membrane. Previous studies on ducts have generally employed only bath perfusion of agonists and other agents, with the result that most receptor systems identified in the duct cells are considered to be present on the basolateral plasma membrane.

The effects of raising luminal $[\text{Ca}^{2+}]_o$, in ducts were in general qualitatively and quantitatively similar to those obtained with Gd$^{3+}$. However, the actions of Ca$^{2+}$, were less clear-cut than in acinar cells, mainly because basolateral (and probably also luminal) $[\text{Ca}^{2+}]_o$, also affects $[\text{Ca}^{2+}]_o$, in ducts via changes in Ca$^{2+}$ entry (16).

**Possible Physiological Role of CaRs in the Pancreatic Duct System**—The unequivocally luminal location of the CaR in the ducts suggests a role for CaRs in the regulation of ductal fluid secretion. This is strongly supported by our experiments showing that luminal Gd$^{3+}$ increased ductal HCO$_3^-$ secretion. Lu-
minal Gd\(^3+\)-stimulated HCO\(_3\)\(^-\) efflux as effectively as forskolin, which we have previously shown to be a potent ductal secretagogue (7, 15).

What might be the physiological significance of the stimulation of ductal secretion by a luminal Ca\(^{2+}\)-sensing receptor? We wish to suggest a possible role in the prevention of pancreatic stone formation. Zymogen granules in acinar cells contain a high concentration of Ca\(^{2+}\) and Mg\(^{2+}\) (32, 33). During exocytosis Ca\(^{2+}\) is therefore released into the lumen, so that pancreatic enzyme secretion is tightly correlated with Ca\(^{2+}\) and Mg\(^{2+}\) release into the duct lumen (28, 34). Excess Ca\(^{2+}\) is potentially dangerous (see Introduction), and we propose that the CaR on the duct cells protects the pancreas by effectively “sensing” the local luminal Ca\(^{2+}\) concentration, and stimulating ductal fluid secretion. Increased ductal fluid secretion would dilute the Ca\(^{2+}\) derived from acinar secretion, thereby preventing precipitation of insoluble Ca\(^{2+}\) salts. A similar role for a luminal CaR in the regulation of fluid transport in the presence of saturating concentration of calcium has been identified in the terminal part of the renal inner medullary collecting ducts, where the receptor may help prevent calcium stone formation by blunting the cellular response to vasopressin (35).

The proposal that ductal fluid secretion is regulated by an apical CaR is supported by the clinical evidence that pancreatitis accompanies familial hypocalciuric hypercalcaemia (36, 37). Familial hypocalciuric hypercalcaemia is an autosomal, dominant disorder associated with an inactivating mutation of the CaR (38). A (partial) loss of CaR function in the duct system of the exocrine pancreas might conceivably, through stone formation, give rise to ductal hypertension or autodigestion (see above), which could account for the pancreatic damage during familial hypocalciuric hypercalcaemia-induced pancreatitis.

**Occurrence of CaR in Islet Cells**—Our immunofluorescence studies also revealed strong staining at the surface of cells in the islets of Langerhans (Fig. 2), consistent with the presence of the receptor in glucagon-secreting α cells and/or insulin-secreting β cells. The presence of the CaR in pancreatic islets, specifically in β cells, has previously been suggested by molecular and functional studies of human insulinoma cells (22, 38). The presence of the receptor in normal rat tissue is interesting, particularly in insuloma cells and/or insulin-sensing receptor? We wonder if the CaR in the ducts can stimulate HCO\(_3\)\(^-\) secretion (28, 34). Excess Ca\(^{2+}\) causes an increase in [Ca\(^{2+}\)]\(_i\) (32, 33). During exocytosis of insulin, the CaR may help prevent calcium stone formation by blunting the cellular response to vasopressin (35).

**Summary**—We have shown that the extracellular Ca\(^{2+}\)-sensing receptor is expressed in acinar and duct cells of the exocrine pancreas and in islet cells. Functional studies of acinar and duct cells confirmed the presence of the receptor, and showed that the receptor in the ducts can stimulate HCO\(_3\)\(^-\) efflux. This strongly suggests that the receptor may have a physiological role in controlling secretion. In the ducts this may be critical in ensuring that fluid secretion is sufficient to prevent the luminal free ionized Ca\(^{2+}\) concentration from rising to levels which precipitate pancreatic stone formation in the presence of the high concentrations of bicarbonate that are typical of the pancreatic juice. In agreement with previous work, the receptor expressed in islet cells probably has a modulatory role in insulin secretion. Further studies are currently in progress to establish the precise nature and cellular distribution of the CaR in the pancreas, as well as the role of the receptor in pancreatic physiological and pathophysiological states.