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A Syndrome of Hypocalciuric Hypercalcemia Caused by Autoantibodies Directed at the Calcium-Sensing Receptor

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Antibodies to cell surface receptors can cause endocrine dysfunction by mimicking or blocking the actions of their respective hormones. We sought patients with autoantibodies to the extracellular calcium (Ca^{2+}_o)-sensing receptor (CaR), which sets the normal level of blood calcium, that mimic the genetic disorder, familial hypocalciuric hypercalcemia, caused by heterozygous inactivating mutations of the CaR. Four individuals from two kindreds were identified with PTH-dependent hypercalcemia, who had other autoimmune manifestations: one with sprue and antigliadin and antiendomyseal antibodies and three with antithyroid antibodies. Three of the patients also had relative or absolute hypocalciuria. The patients' sera contained antibodies that reacted with the cell surface of bovine parathyroid cells in a manner similar to an authentic polyclonal anti-CaR antibody, stained bands on Western analysis of sizes similar to those labeled by the anti-CaR antiserum, and reacted with several synthetic peptides derived from sequences within the CaR's extracellular amino terminus. The patients' sera also stimulated PTH release from dispersed human parathyroid cells compared with the effect of sera from normocalcemic control subjects. This stimulation could be blocked by preabsorbing serum with membranes from CaR-transfected, but not nontransfected, human embryonic kidney (HEK293) cells. Finally, in two of the patients, antibodies affinity-purified using a synthetic peptide from within the CaR's extracellular domain inhibited high Ca^{2+}_o-stimulated, CaR-mediated accumulation of inositol phosphates and activation of mitogen-activated protein kinase in CaR-transfected HEK293 cells. DNA sequencing revealed no mutations within the index patients' CaR genes in the two families. Therefore, a biochemical phenotype of PTH-dependent hypercalcemia resembling that caused by heterozygous inactivating mutations of the CaR in familial hypocalciuric hypercalcemia can be observed in patients with antibodies to the CaR's extracellular domain that stimulate PTH release, probably by inhibiting activation of the CaR by Ca^{2+}_o. Autoimmune hypocalciuric hypercalcemic is an acquired disorder of Ca^{2+}_o sensing that should be differentiated from that caused by inactivating mutations of the CaR. (*J Clin Endocrinol Metab* 88: 60–72, 2003)

FAMILIAL HYPOCALCIURIC hypercalcemia (FHH; also referred to as familial benign hypercalcemia) was originally described in 1966 as a variant of hyperparathyroidism (1, 2). This disorder, which was subsequently shown to be distinct from primary hyperparathyroidism, is characterized by mild to moderate hypercalcemia in the setting of relative hypocalciuria and inappropriately normal or only slightly elevated circulating levels of PTH (3–5). From the outset, it was recognized to be an inherited syndrome with an autosomal dominant pattern of inheritance (3, 4). In an individual patient, it can be difficult to distinguish FHH from mild primary hyperparathyroidism (PHPT) (6). However, this distinction is important, because FHH generally does not predispose to the usual symptoms and complications of hyperparathyroidism, and parathyroid surgery is typically ineffective in correcting the hypercalcemia (3–5, 7).

On the average, patients with FHH have much lower rates of urinary calcium excretion than those with similar degrees of elevation of serum calcium concentration due to PHPT (3, 4). There is some overlap, however, in the values for urinary calcium excretion in these two groups of patients, when calcium excretion is expressed in absolute terms (*e.g.* as milligrams per 24 h) or as the urinary calcium to creatinine clearance ratio (3). Therefore, a secure diagnosis of FHH has generally required demonstration of the expected biochemical phenotype combined with an autosomal dominant pattern of hypercalcemia in the patient's family (5).

Early investigations of the pathophysiology of FHH proposed that the disorder resulted from inappropriate sensing/handling of extracellular calcium (Ca^{2+}_o) by the parathyroid glands and kidney (2, 3). This concept has subsequently been proven to be correct, and FHH is now known to be caused in the vast majority of cases by mutations in the extracellular calcium-sensing receptor (CaR) gene, which resides on the

Abbreviations: Ca^{2+}_o, Extracellular calcium; CaR, calcium-sensing receptor; ERK, extracellular signal-regulated kinase; FHH, familial hypocalciuric hypercalcemia; MAPK, mitogen-activated protein kinase; PHPT, primary hyperparathyroidism; TCA, trichloroacetic acid.

long arm of chromosome 3 (5, 8–10). In two kindreds, the clinical syndrome of FHH has been mapped to the short (11) or the long (12) arm of chromosome 19, respectively. The CaR is a G protein-coupled receptor that responds to Ca^{2+}_o as its principal physiological ligand (13). It was the first such receptor described that recognizes an inorganic ion as its primary physiological ligand (14). This protein is the critical sensor by which the parathyroid gland regulates the secretion of PTH in response to changes in the blood level of Ca^{2+}_o. The kidney uses the CaR to directly sense Ca^{2+}_o and regulate renal Ca^{2+}_o handling in an appropriate manner, increasing Ca^{2+} excretion when Ca^{2+}_o is high and reducing it when Ca^{2+}_o is low, independently of concomitant CaR-mediated changes in the level of circulating PTH (15).

In both humans with FHH (16) and a mouse model heterozygous for targeted inactivation of the CaR gene (17), there is a biochemical phenotype of hypocalciuric hypercalcemia due to the loss of one normal allele of the CaR gene. The resultant reduction in expression of the normal CaR protein in parathyroid and kidney causes resistance of these tissues to Ca^{2+}_o, thereby increasing PTH secretion and decreasing renal Ca^{2+}_o excretion so as to reset Ca^{2+}_o to an elevated level. The Ca^{2+}_o homeostatic system then achieves a new steady state characterized by hypercalcemia, abnormally high (*e.g.* inappropriate for the prevailing serum Ca^{2+}_o concentration) levels of PTH, and relative hypocalciuria, all hallmarks of FHH. In further support of this interpretation, homozygous loss of function mutations of this gene result in a more severe form of Ca^{2+}_o resistance and hypercalcemia, known as neonatal severe hyperparathyroidism (16, 18). In contrast, gain of function mutations cause hypocalcemia and relative or absolute hypercalciuria (19, 20), thereby causing a form of hypoparathyroidism that can occur sporadically or as an autosomal dominant disorder.

We recently encountered several patients with PTH-dependent hypercalcemia in the setting of conditions thought to be caused on an autoimmune basis (*e.g.* nontropical sprue or Hashimoto's thyroiditis). We tested the hypothesis, therefore, that these patients had autoimmune FHH due to the presence of autoantibodies partially interfering with the normal activation of the CaR by hypercalcemia. These patients in most cases exhibited not only PTH-dependent hypercalcemia, but also relative or absolute hypocalciuria (*e.g.* the cardinal biochemical features of FHH), presumably because these antibodies bind to and partially inactivate the CaR in parathyroid and kidney. Therefore, a syndrome closely resembling FHH in its biochemical features can be produced by anti-CaR antibodies and should be considered in the differential diagnosis of PTH-dependent hypercalcemia. Because this condition is presumably acquired, rather than being present from the time of conception, as is the case in FHH, the optimal management of the signs and symptoms that may arise from hypercalcemia and/or relative or absolute PTH excess in this autoimmune condition remains to be determined.

Subjects and Methods

Family 1

A 19-yr-old Bolivian woman (patient 1-1) presented to the Yale Bone Center for a second opinion regarding a recommendation for parathyroidectomy. She had been well until age 14 yr, when she was diagnosed as having Hashimoto's thyroiditis and was started on therapy with L-T_4. At age 18 yr, she was noted to have an elevated serum calcium level of 3.05 mmol/liter on routine biochemical screening. A repeat calcium level was 2.88 mmol/liter, and an intact PTH level was 40 pg/ml (normal, 10–65). A sestamibi scan suggested the presence of a left inferior parathyroid adenoma. She was given a diagnosis of primary hyperparathyroidism, and surgery was recommended, but the patient sought another opinion.

Upon presentation to the Yale Bone Center, she denied most of the usual symptoms of hypercalcemia. She complained of mild polydipsia and polyuria, however, although she did not experience nocturia. Her past medical history was remarkable for hepatitis A at age 4 yr and a 2-yr history of irregular menses associated with vigorous exercise and weight loss. She was a college sophomore and lived in Bolivia when not attending college. Her family history was strongly positive for Hashimoto's thyroiditis, and her younger sister had recently been diagnosed with hypothyroidism and hypercalcemia. She was not aware of any diseases of calcium metabolism in other members of her family. Her physical examination was unremarkable. Biochemical testing revealed elevated total serum calcium levels ranging from 2.68–2.95 mmol/liter, a normal serum phosphorus concentration of 1.06 mmol/liter, and normal serum levels of albumin, electrolytes, blood urea nitrogen, and creatinine (summarized, along with the respective normal ranges, in Table 1). Her PTH levels were 42 nl equivalents/ml (normal, <25 nl equivalents/ml) in a midregion assay when her serum calcium concentration was 2.95 mmol/liter and 66 pg/ml in an intact PTH assay (normal, 10–65 pg/ml) when her serum calcium concentration was 2.75 mmol/liter. Her 25-hydroxyvitamin D level was normal at 52.4 nmol/liter, and her 1,25-dihydroxyvitamin D level was at the upper limit of normal at 149 pmol/liter. Her thyroid hormone, TSH, and PRL levels were normal. Her 24-h urinary calcium excretion was measured on two occasions. The first result was 1.43 mmol and corresponded to a calcium to creatinine clearance ratio of 0.006. On the second occasion, 2 months later, it was 3.43 mmol, and the calcium to creatinine clearance ratio was 0.013. A diagnosis of FHH was suspected, and her family was screened for hypercalcemia. A pedigree for the family is shown in Fig. 1 (see *Results*). Her sister (patient 1-2, mentioned above), a paternal uncle, and her father were found to be mildly hypercalcemic. The proband, her sister, and their father had antithyroid antibodies, and a paternal uncle and a paternal aunt had hypothyroidism without detectable antithyroid antibodies (Fig. 1). Serum samples for the studies carried out here were obtained from this sister, but not from the father. Her mother and another sister had normal serum calcium concentrations. She was given a diagnosis of FHH, and surgery was not performed. She has remained hypercalcemic, but otherwise well, except for mild symptoms of polyuria and polydipsia.

Family 2

A 47-yr-old female (patient 2-1) presented in 1996 with weight loss, anorexia, diarrhea, diffuse abdominal pain, and iron deficiency anemia. An initial evaluation revealed an elevated serum antigliadin immunoglobulin (Ig) level (Table 1) of 90 (normal, 0–42) as well as the presence of antiendomyseal antibodies (IgA), duodenitis by endoscopy, and biopsy-confirmed celiac sprue. Despite moderate compliance with a gluten-free diet, she still complained in January 1997 of diffuse abdominal pain, deep bony pain in her forearms and legs, and generalized muscle aches. Other medical history included contact dermatitis, a history of a traumatic pelvic fracture, degenerative joint disease, anxiety, depression, migraine headaches, fibrocystic breast disease, and atypical chest pain. Her medications included Zantac, Naprosyn, ferrous sulfate, and Premarin.

Her serum total calcium concentration was 2.88 mmol/liter, with a concomitant intact PTH of 27 pg/ml (see Table 1 for a summary of laboratory values and respective normal ranges). Complete blood count showed microcytic anemia with a hematocrit of 33.5%, a mean corpuscular volume of 71.5, and a low serum iron level. Serum chemistries were normal, including alkaline phosphatase of 87 U/liter, phosphorus of 1.03 mmol/liter, magnesium of 1.0 mmol/liter, erythrocyte sedimentation rate of 15, and TSH of 3.5. Serum protein electrophoresis, serum immunoelectrophoresis, and urinary protein electrophoresis were all normal. A screen for autoantibodies (Quest Diagnostics, Inc., Norwich, NY)

TABLE 1. Table of chemistries for affected patients

Patient no.	Calcium (2.15–2.6 mmol/liter)	Intact PTH (10–65 pg/ml)	Phosphorus (0.7–1.5 mmol/liter)	Magnesium (0.66–1.1 mmol/liter)	Creatinine (44–115 μmol/liter)	Urinary calcium (1.25–10 mmol/24 h)	25 Vitamin D (47–130 nml/liter)	1,25 Vitamin D (36–144 pmol/liter)
1-1								
3/98	2.88	40	0.84	1.0			54.9	149
4/98	2.75	66	1.06			1.43		
6/98	2.95		0.93			3.43	53.4	148
11/98	2.68		0.8					
4/99	2.58	18	1.06					
1–2								
4/98	2.63		1.06					
5/98	2.8		1.16					
6/16/98	2.58	18	1.03			1.05	60.7	143
2-1								
1/29/97	2.88	27		1.2		0.6		202
5/1/97	2.63	39		1.1		2.6	25	
Pre-op								
Post-op	2.38	32.5					57.4	134
5/7/97	2.68	35						
1/29/99	2.65	35					92.4	
10/24/99	2.68		1.0					
2/10/00	2.7	41		0.95	44.2		59.9	
2-2								
1/26/98	2.83		0.68					
2/2/98	2.78	23	1.03	0.95		6.13		139
2/6/98	2.6	41	1.06					
3/1/2000	2.78	49						

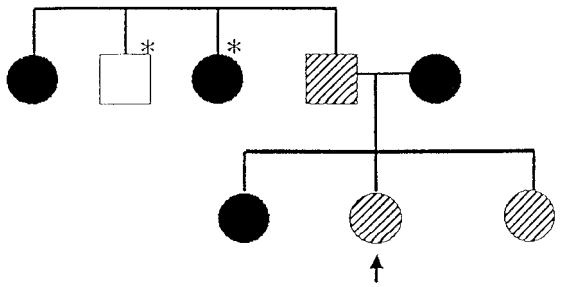

FIG. 1. Pedigree of family 1 showing the pattern of hypercalcemia and thyroiditis. □, Hypercalcemia; ■, family members with normal calcium levels; ▨, family members with both hypercalcemia and documented Hashimoto's thyroiditis. Family members with *asterisks* are hypothyroid, but do not have antithyroid antibodies. The proband is denoted by the *arrow*.

revealed an elevated anti-LA antibody of 11 U/ml (normal, 0–3 U/ml) and a speckled antinuclear antibody titer of 1:80 (borderline high); all other antibodies were normal, including anti-double-stranded DNA, anti-single-stranded DNA, anti-smooth muscle, antiribonuclear protein, and anti-RO, as were C3 and C4 complement levels. CH_{50} was 160. Twenty-four-hour urinary calcium excretion was 0.6 mmol, with a creatinine clearance of 82 ml/min, consistent with malabsorption due to celiac sprue. Supplementation with a high calcium diet (1000 mg elemental calcium/d) increased urinary calcium excretion to 2.63 mmol/24 h. A calcium to creatinine ratio was not determined. Bone scan revealed diffusely increased uptake in the sternum and spine, suggestive of hyperparathyroid bone disease. Bone mineral density showed mild osteopenia of the lumbar spine (T score, –1.05). An ultrasound of the neck revealed a 0.7 × 0.4-cm hypoechoic area in the left lower neck, consistent with a left inferior parathyroid adenoma. A sestamibi scan, however, showed no persistent area of increased uptake that would have been consistent with an enlarged parathyroid gland.

In May 1997, her symptoms and biochemical findings were attributed to hyperparathyroidism, including calcium and intact PTH concentrations of 2.63 mmol/liter and 39 pg/ml, respectively, determined at that time. Surgical exploration of the neck revealed a normal-appearing left inferior parathyroid. A modestly enlarged left upper parathyroid gland was removed. Pathological examination revealed a 1.0 × 0.7-cm hypercellular parathyroid gland containing 30–40% fat with fibrous tissue adjacent to the parathyroid gland, consistent with the presence of a small adenoma. Postoperatively, the serum calcium concentration decreased to 2.38 mmol/liter with a concomitant intact PTH level of 32.5 pg/nl. One week later, the serum total calcium concentration increased to 2.68 mmol/liter with an intact PTH level of 35 pg/ml. The patient's symptoms did not improve. Later in 1997, an ultrasound and magnetic resonance imaging of the neck showed a small nodule posterior to the left lower pole of the thyroid, consistent with a small parathyroid adenoma. A selective angiogram of the neck with venous sampling, performed in November 1997, revealed a moderate elevation in the level of PTH in the thyroid venous drainage in the absence of definitive angiographic evidence for a parathyroid adenoma. An attempt at angiographic ablation of a presumed parathyroid adenoma was unsuccessful. In January 1999, her serum calcium and intact PTH levels were 2.65 mmol/liter and 35 pg/ml, respectively. She continues to have anxiety, depression, generalized aches and pains, and a sensation of deep bony pain. Her serum calcium ranges between 2.63–2.7 mmol/liter with concomitant intact PTH levels of 35–41 pg/ml and a normal level of 25-hydroxyvitamin D (59.9 nmol/liter). Treatment with infusion of a bisphosphonate (pamidronate) every 3 months for a presumed diagnosis of osteoporosis has not relieved her bony symptoms.

The 23-yr-old daughter (patient 2-2) of patient 2-1 has a history of diffuse musculo-skeletal aches and pains as well as an anxiety disorder. She has no history of abdominal symptoms, kidney stones, or bone pain and takes no medications. On biochemical screening, her serum calcium was 2.83 mmol/liter with an intact PTH of 23 pg/ml, phosphorus of 1.03 mmol/liter, magnesium of 0.68 mmol/liter, albumin of 4.2 g/dl, and 24-h urinary calcium excretion of 6.13 mmol. She had an elevated level of thyroid peroxidase antibodies of 22 IU/ml (normal, 0–1.9; see Table 1) and a mildly elevated level of anti-smooth muscle antibodies (12 U/ml). Screening for other autoantibodies was negative, including antigliadin and antiendomyseal antibodies. The family refused any further family screening.

Informed consent was obtained from the patients; the evaluation at Brigham and Women's Hospital was reviewed by the human research committee, and, in accordance with institutional policy, was approved as an acceptable innovative approach to clinical diagnosis or therapy. Studies performed on patients evaluated at Yale Bone Center were approved by the Yale University human investigation committee.

Materials and Methods

Materials

Polyclonal rabbit anti-CaR antisera 4641 (raised against amino acids 214–238 in the human CaR) and 4637 (raised against amino acids 344–358 in the human CaR) and monoclonal anti-CaR antibody LRG (raised against amino acids 374–391 in the human CaR) were gifts from Drs. Kim Rogers and Edward Nemeth (NPS Pharmaceuticals, Inc., Salt Lake City, UT) and Drs. Allen Spiegel and Paul Goldsmith (NIDDK, NIH, Bethesda, MD).

Specimen collection and preparation

Serum samples were collected and stored at –20 or –80 C. Purified IgG were isolated using rProtein G agarose (Life Technologies, Inc., Gaithersburg, MD). Sera were affinity-purified using a peptide corresponding to amino acid residues 344–358 of the human CaR, which had been used to raise and then affinity-purify rabbit polyclonal antiserum 4637 (21) (see below).

Affinity purification of sera and antisera

Sera from the four hypercalcemic patients, sera from normocalcemic controls, or rabbit polyclonal anti-CaR antiserum 4637 were affinity-purified in the following manner. The affinity columns were made up by coupling the peptide under anhydrous conditions to Affi-Gel 10 (activated immunoaffinity support, Bio-Rad Laboratories, Inc., Hercules, CA). The sera or antiserum were absorbed on the affinity column in PBS, eluted with 20 mM HCl (pH 2.5), and immediately neutralized as recommended by Bio-Rad Laboratories, Inc. The purified antibodies were stored at –20 C until use.

Immunoperoxidase and immunofluorescence

Frozen sections of bovine parathyroid glands or dispersed bovine parathyroid cells (22) were fixed in PBS containing 4% paraformaldehyde at 20 C for 10 min. Immunoperoxidase staining was performed using patient or control sera (1:100 dilution), purified Ig (1:100 dilution), or an affinity-purified, polyclonal anti-CaR antiserum (no. 4637) (23). Bound Ig were detected using a peroxidase-conjugated, goat antihuman antiserum specific for the μ-chain of IgM or a goat antihuman antiserum specific for the γ-chain of IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or a peroxidase-conjugated goat antirabbit antiserum (Sigma-Aldrich, St. Louis, MO), respectively, and the DAKO Corp. (Carpinteria, CA) 3-amino-9-ethyl-carbazole substrate system (21).

Two-color immunofluorescence to detect colocalization of anti-CaR antibodies in sera with anti-CaR antiserum 4637 was performed as previously described (21). In brief, parathyroid cells were incubated with both antiserum 4637 (1:200 dilution) and patient or control serum (1:100 dilution), and then with secondary antibodies (goat antirabbit IgG tagged with Alexa 568, Molecular Probes, Inc., Eugene, OR) and a fluorescein-conjugated goat antihuman antiserum specific for the γ-chain of IgG. Fluorescence images were collected using an MRC 1024/2P multiphoton microscope (Bio-Rad Laboratories, Inc.) at Brigham and Women's Hospital Confocal Facility.

Western analysis

Proteins extracted from bovine parathyroid or CaR-transfected human embryonic kidney (HEK293) cells were separated by PAGE and transferred to nitrocellulose membranes (24). The membranes were then incubated with affinity-purified antiserum 4637 (1:1000), or patient or control serum that had been affinity-purified against the peptide used to raise antiserum 4637, followed by horseradish peroxidase-conjugated goat antirabbit or antihuman antisera.

Immunoprecipitation

CaR-transfected HEK (HEKCaR) cells were washed with ice-cold PBS and lysed with immunoprecipitation buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM sodium *o*-vanadate, protease inhibitors (24), and 1% Triton X-100. The cell lysates were centrifuged at 10,000 $\times$ *g* for 10 min. Supernatant protein (500 μg total lysate) were incubated with affinity-purified serum prepared from the four patients' sera or with monoclonal anti-CaR antibody (LRG) overnight at 4 C. Protein A/G-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were then added for an additional 1 h at 4 C. Bound immunocomplexes were washed three times with immunoprecipitation buffer, and the pellet was eluted by boiling for 5 min with 2$\times$ Laemmli sample buffer. After SDS-PAGE, Western blot analysis was performed as described above using affinity-purified polyclonal anti-CaR antiserum 4637.

Reactivity of anti-CaR antibodies with synthetic peptides

Immulon II microtiter plates (Dynatech Corp., Chantilly, VA) were incubated overnight at 4 C with 1–5 μg peptide in carbonate buffer, pH 9.6, and washed three times. Patient or control serum (diluted 1:1000 in PBS/0.05% Tween 20) were added and incubated at 37 C for 1 h. After washing and incubation with 50 μl/well of a peroxidase-conjugated goat antihuman antiserum specific for the γ-chain of IgG overnight at 4 C, immunoreactivity was quantified using an ML3000 Microtiter Plate Luminometer (Dynatech Corp.).

Preparation of membranes from CaR-transfected or nontransfected HEK293 cells

Confluent monolayers of HEK293 cells stably transfected with the CaR or nontransfected HEK293 cells (25) were rinsed with ice-cold PBS and scraped into lysis buffer [10 mmol/liter Tris-HCl (pH 7.4), 1 mmol/liter EDTA, 1 mmol/liter EGTA, 0.25 mol/liter sucrose, 1 mmol/liter dithiothreitol, and protease inhibitors] (24, 26). The cells were passed through a 22-gauge needle 10 times, and nuclei and cellular debris were removed by sedimentation. Supernatant volumes were adjusted to equalize protein concentrations, the supernatants were sedimented at 45,000 $\times$ *g* for 1 h, and the pellets were resuspended in Eagle's MEM containing leupeptin and calpain inhibitor. Resuspended membranes (20 μg protein) were incubated with 100 μl patient or control serum in 500 μl Eagle's MEM for 1 h at 37 C. As additional controls, sera were incubated similarly except without membranes. After the incubations, sera were sedimented, and supernatants were diluted with Eagle's MEM (2% final concentration) and saved for incubation with parathyroid cells (see below).

Determination of Ca^{2+}_o-regulated PTH release

In most experiments dispersed human parathyroid cells (1×10^6 cells/0.5 ml) (27) were preincubated for 1 h at 37 C with 2% (vol/vol) patient or control serum, washed with Eagle's MEM, and incubated with 0.5 mmol/liter Mg^{2+}_o and varying concentrations of Ca^{2+}_o (0.5–3.0 mmol/liter) for 1 h at 37 C in Eagle's MEM with 2% serum (vol/vol) from the same patient or control used during the preincubation. In a second experimental design (see *Results*), cells were preincubated, washed, and incubated as described above, except with IgG purified from patient or control serum (1:100 dilution) rather than with 2% sera. In a third experimental design, cells were preincubated, washed, and incubated with 2% patient or control sera preabsorbed with membranes from CaR-transfected or nontransfected HEK293 cells. Supernatant PTH was measured using the Whole PTH (1–84) Specific Immunoradiometric Assay kit (Scantibodies Laboratory, Inc., Santee, CA) (28, 29).

Determination of CaR-stimulated inositol phosphate accumulation

Cells prelabeled with [^{3}H]myo-inositol were incubated with variable concentrations of $CaCl_2$ for 30 min in the presence of 10 mM LiCl (25) after preincubation with affinity-purified sera pooled from three normocalcemic controls or from patients 2-1 or 2-2 or with anti-CaR antiserum LRG. The reactions were terminated with 10% ice-cold trichloroacetic acid (TCA; final wt/vol). After centrifugation to remove insoluble debris, TCA was extracted with water-saturated diethyl ether, and inositol phosphates were separated on Dowex anion exchange columns (BioRad, Hercules, CA) as described previously and quantitated using a liquid scintillation counter (25).

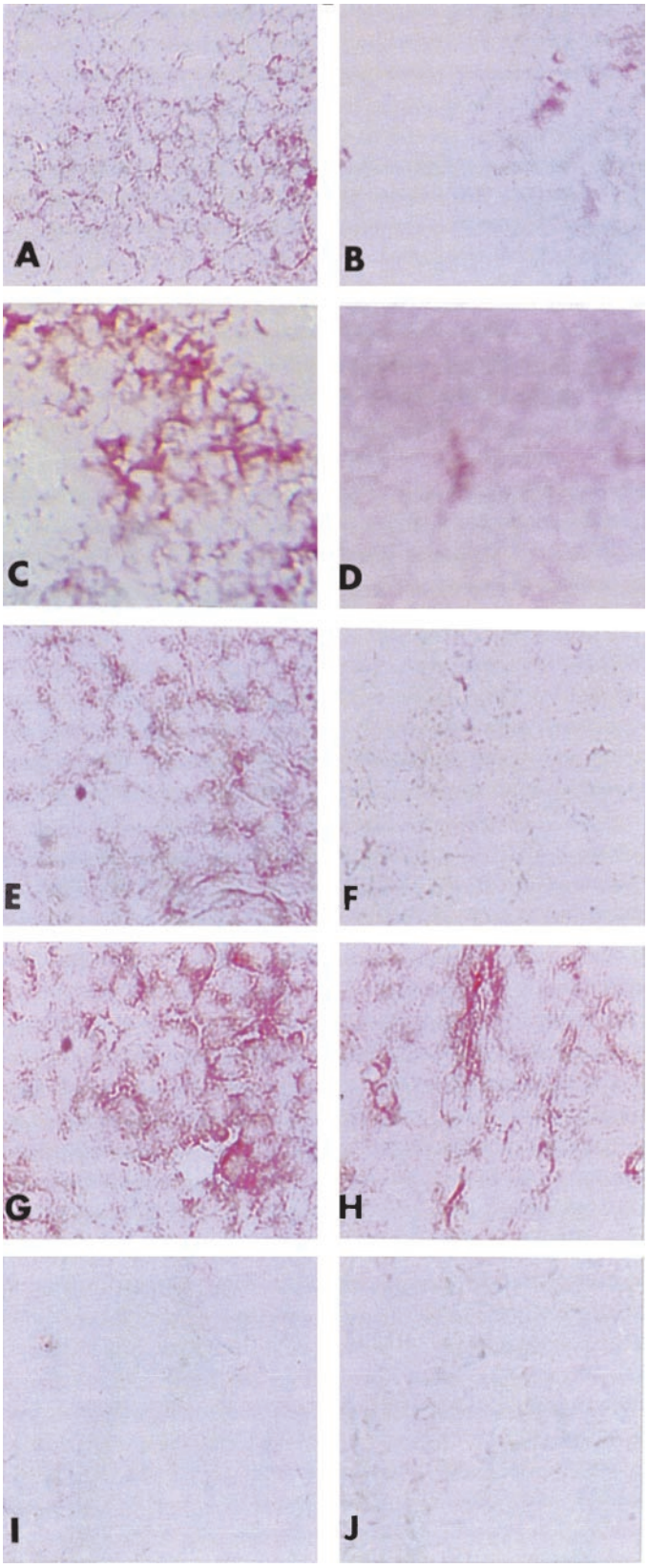

FIG. 2. Immunoperoxidase staining of bovine parathyroid gland sections by sera from patients 1-1 (A and B), 1-2 (C and D), 2-1 (E and F), and 2-2 (G and H) compared with sera pooled from five control normocalcemic subjects (I and J). Frozen sections were incubated with the respective sera and then with secondary antisera [a peroxidase-conjugated, goat antihuman antiserum specific for the γ-chain of IgG (A, C, E, G, and I) or a peroxidase-conjugated goat antihuman antiserum specific for the μ-chain of IgM (B, D, F, H, and J)], as described in *Materials and Methods*. Note the staining of the parathyroid cell surface by IgG from the four patients and the staining of endothelial cells in parathyroid vasculature by IgM from patient 2-2. The pooled control sera produced little or no staining. Photomicrographs were taken at ×400.

Determination of CaR-stimulated mitogen-activated protein kinase (MAPK) activity

For the determination of extracellular signal-regulated kinase (ERK1/2) phosphorylation, which reflects activation of these MAPKs, monolayers of serum-starved HEKCaR cells were preincubated with affinity-purified control serum (pooled from three normocalcemic controls), affinity-purified serum from patient 2-1 or 2-2, or affinity-purified antiserum 4637 and then incubated at 37 C in serum-free medium containing 0.2% BSA with varying concentrations of Ca^{2+}_o. At the end of the incubation period, the medium was removed, the cells were washed with ice-cold PBS containing 1 mM sodium vanadate, cells were lysed directly with 100 μl/well Laemmli sample buffer, cell lysates were sonicated briefly, and proteins were resolved by SDS-PAGE. The separated proteins were transferred electrophoretically onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) and incubated with blocking solution [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20] containing 5% dry milk for 1 h at room temperature. ERK1/2 phosphorylation was detected by immunoblotting using an overnight incubation with a 1:1000 dilution of a rabbit polyclonal phospho-ERK1/2-specific antiserum and a subsequent incubation with a second goat antirabbit, peroxidase-linked antiserum diluted in blocking solution. The bands were visualized by chemiluminescence system (Renaissance ECL system; New England Nuclear, Boston, MA). Quantitative comparisons of the phosphorylation of ERK1/2 under various experimental conditions were performed using ImageQuant and a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA). Nitrocellulose membranes were stripped of antibodies and reprobed using an anti-ERK2 monoclonal antibody (Transduction Laboratories, Inc., Lexington, KY) that detects this protein independent of its state of phosphorylation to confirm equal loading of ERK protein. Protein concentrations were measured using the Micro BCA protein kit (Pierce Chemical Co., Rockford, IL).

DNA sequencing

The coding exons of the CaR genes from blood cells of patients 1-1 and 2-1 were amplified by PCR and sequenced (10, 30). The sequences were compared with those amplified from control DNA and with the human CaR cDNA (accession no. U20759) (31).

Statistical analyses

Data represent the mean ± SEM. Statistical analyses were performed with unpaired *t* test when two groups were compared or with ANOVA when three or more groups were compared or multiple comparisons were carried out with a single control. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical features of the four patients

Table 1 summarizes the biochemical features of the four hypercalcemic patients in the two families that studied here; their clinical and biochemical features and clinical courses are described in greater detail in the case reports of *Subjects and Methods*. All four had mild to moderate hypercalcemia accompanied by inappropriately normal (*i.e.* nonsuppressed) PTH levels. In three of the four patients, urinary calcium excretion, expressed either as 24-h

excretion rate or as the ratio of the renal clearance of calcium to that of creatinine, was inappropriately normal or frankly low, in the range encountered in patients with FHH on a genetic basis. Figure 1 shows a pedigree for family 1, indicating those individuals with hypercalcemia and/or hypothyroidism with or without antithyroid antibodies. Only the proband and her daughter could be studied in family 2.

Patients' sera contain antibodies binding to parathyroid cells

Figure 2 shows that IgG and/or IgM in sera from patients 1-1, 1-2, 2-1, and 2-2, but not in pooled control sera, bound to bovine parathyroid cells with a pattern consistent with binding to the cell surface. Serum from patient 2-2 also stained endothelial cells. IgG purified from the sera of patients 1-2 and 2-1, but not that from pooled control sera, colocalized on the parathyroid cell surface with an affinity-purified, polyclonal anti-CaR antiserum raised in a rabbit (4637; Fig. 3), as would be expected if the patient serum contained anti-CaR antibodies.

Patients' sera contains anti-CaR antibodies by Western analysis

To obtain more definitive evidence that the patients' sera contained anti-CaR antibodies, we performed Western analysis using sera from all four patients that had been affinity-purified using the same peptide employed to raise polyclonal anti-CaR antiserum 4637 to reduce nonspecific staining resulting from the use of whole serum. These affinity-purified antibodies showed labeling of bands of sizes consistent with CaR monomers and dimers glycosylated to varying extents based on their being of similar or identical sizes to the bands in extracts of bovine parathyroid glands that were labeled using a polyclonal anti-CaR antiserum (Fig. 4A). The labeling of these bands by antibodies affinity-purified from patient 2-2's serum was abolished by preincubation with the peptide used for affinity purification, strongly suggesting that they were specific for this epitope of the CaR (Fig. 4A).

To provide additional evidence for the presence of anti-CaR antibodies in the patients' sera, affinity-purified Ig from each of the four patient's serum as well as from control serum were used to immunoprecipitate proteins extracted from CaR-transfected HEK cells. Immunoprecipitation was also performed with anti-CaR antiserum LRG as a positive control. The immunoprecipitated proteins were then subjected to Western blotting using anti-CaR antiserum 4637. Figure 4B shows that a doublet of bands between the 127,000 and 217,000 molecular weight markers, similar in molecular weight to the CaR-specific bands detected in Fig. 4A by direct Western blotting, were immunoprecipitated by the four pa-

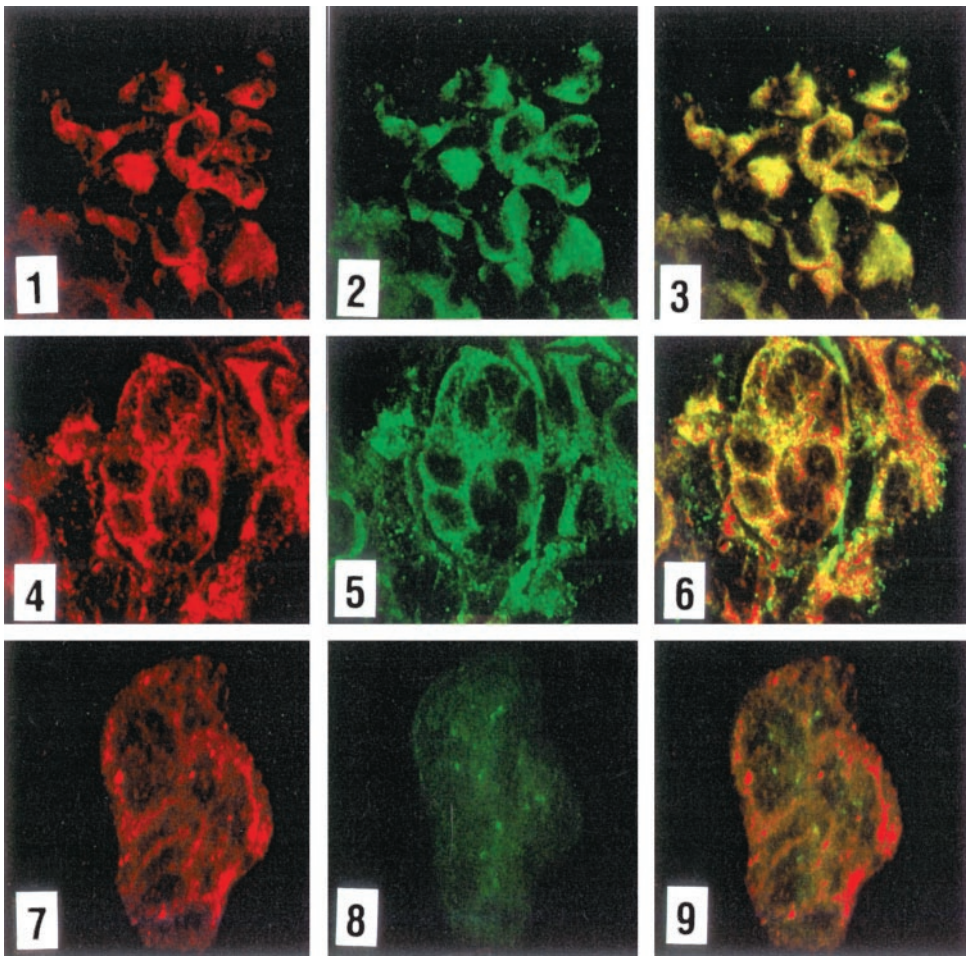

FIG. 3. Colocalization of IgG from patients 1-2 and 2-1 with affinity-purified polyclonal anti-CaR antiserum 4637 in bovine parathyroid cells. Dispersed bovine parathyroid cells were immunostained using the immunofluorescence technique and confocal microscopy as described in *Materials and Methods* with anti-CaR antiserum 4637 (*red* in panels 1, 4, and 7), sera of patients 1-2 (panels 1–3) and 2-1 (panels 4–6), or sera pooled from five normocalcemic control sera (panels 7–9). Merging of the images in panels 1 and 2 (in panel 3) and in 4 and 5 (in panel 6) shows extensive colocalization (*yellow*) of anti-CaR antiserum 4637 with IgG in patients 1-2 and 2-1. In contrast, anti-CaR antiserum 4637 does not significantly colocalize with IgG in the pooled control sera (note lack of *yellow* color in panel 9). Photomicrographs were taken at ×1000.

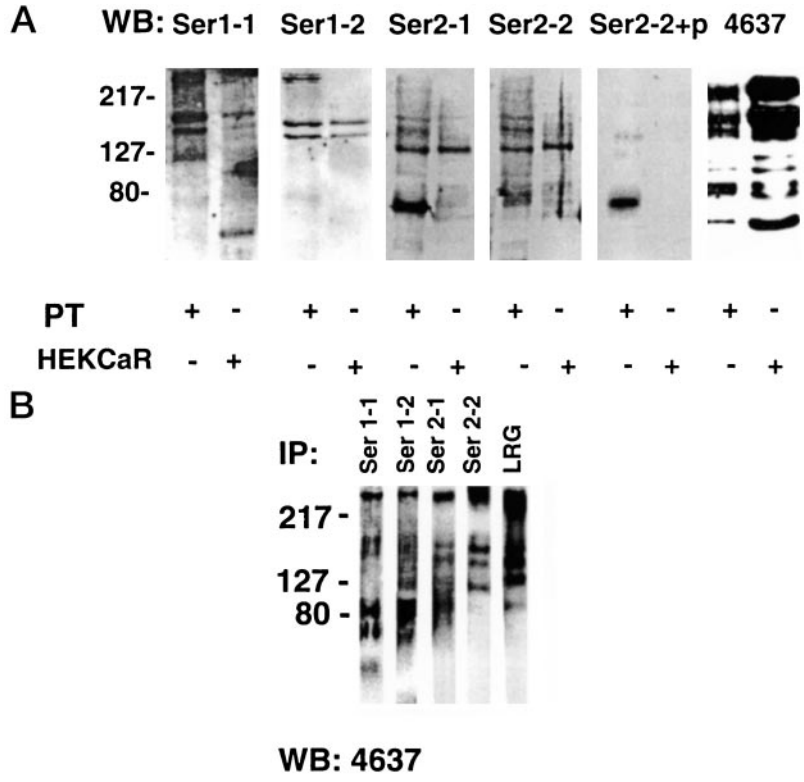

FIG. 4. Western blots of proteins prepared from bovine parathyroid gland (Pt.) or HEKCaR cells using affinity-purified serum from the patients' sera or affinity-purified rabbit polyclonal antiserum 4637 (A). The results show Western blots obtained using affinity-purified serum from patient 1-1 (Ser1-1), 1-2 (Ser1-2), 2-1 (Ser2-1), or 2-2 (Ser2-2) prepared as described in *Materials and Methods* as well as affinity-purified serum from patient 2-2 after preabsorption with the same peptide used for affinity purification (Ser2-2 + p). The result of Western analysis carried out similarly, except with affinity-purified antiserum 4637, is shown for comparison (4637). Protein samples (20 μg in each lane) were subjected to PAGE and electrotransferred to nitrocellulose blots as described in *Materials and Methods*. After incubation with affinity-purified patient serum or antiserum 4637, the blots were washed, and bound antibodies were identified using a goat antihuman antiserum specific for the γ-chain of IgG as described in *Materials and Methods*. Several Western blots were carried out with similar results. All bands above 80K were abolished after pretreatment of antiserum 4637 with the peptide against which it was raised (not shown, see Ref. 23) or after similar preincubation of affinity-purified serum from patient 2-2 with the same peptide. B, Results of Western blotting of proteins immunoprecipitated from lysates of HEKCaR cells with affinity-purified sera from the four patients (first four lanes), monoclonal anti-CaR antibody LRG (lane 5), or a pool of affinity-purified normocalcemic control sera (lane 6). After separation of proteins by SDS-PAGE, Western analysis was performed as in A, using anti-CaR antiserum 4637 as described in *Materials and Methods*.

tients' affinity-purified sera as well as by anti-CaR antiserum LRG, but not by affinity-purified control serum.

Purified Ig in patients' sera bind CaR peptides

To document further that the anti-CaR antibodies in the patients' sera recognized epitopes present within the CaR's extracellular amino terminus, we evaluated the capacity of protein G-purified IgG from their sera to bind to three synthetic peptides with sequences corresponding to residues 214–236 (4641 peptide), 344–358 (4637 peptide), and 374–391 (LRG peptide) of the human CaR. Figure 5, A and B, reveals that the four patients' purified IgG showed substantially greater binding to these three synthetic peptides than did purified IgG from normocalcemic control subjects. The binding of IgG purified from 13 normocalcemic patients' sera who had antithyroid antibodies (antithyroid peroxidase and/or antithyroglobulin) was similar to that of normocalcemic controls (Fig. 5C). Thus, autoantibodies to the CaR are not common in patients with another form of autoimmunity.

Inhibition of high Ca^{2+}_o-stimulated, CaR-mediated accumulation of inositol phosphates and activation of ERK1/2 MAPK by anti-CaR antibodies in HEKCaR cells

To investigate whether the patients' anti-CaR antibodies blocked activation of the receptor by high Ca^{2+}_o, we tested the effect of affinity-purified sera from patients 2-1 and 2-2 on high Ca^{2+}_o-stimulated accumulation of inositol phosphates (25) and activation of MAPK (32) as indexes of receptor-mediated activation of CaR-regulated intracellular signaling pathways in CaR-transfected HEK293 cells. Figures 6 and 7 show that antibodies purified from both patients' sera inhibited high Ca^{2+}_o-stimulated accumulation of total inositol phosphates and activation of ERK1/2, respectively, relative to what was observed with affinity-purified serum from several control subjects. The monoclonal anti-CaR antiserum raised against the LRG peptide and the affinity-purified rabbit polyclonal antiserum raised against the 4637 peptide inhibited total inositol phosphate generation and MAPK activation, respectively, to extents similar to those observed with the two patients' affinity-purified antibodies.

Patients' sera stimulate PTH release

Because normal human parathyroid cells were unavailable, we used parathyroid cells from adenomas or hyperplastic parathyroid glands resected from patients with hyperparathyroidism to study the effects of the patients' sera on Ca^{2+}_o-regulated PTH release. Such pathological parathyroid glands express the CaR, albeit at subnormal levels (23, 33, 34), and generally retain responsiveness to Ca^{2+}_o (35). Preincubating parathyroid cells with 2% serum from all four patients stimulated PTH release over that observed after preincubation with control serum (Fig. 8) at 0.5–2.0 mM Ca^{2+}_o. IgG purified from the serum of patient 2-1 also stimulated PTH release over that seen with control IgG, supporting an antibody-mediated effect (Fig. 9A). Preabsorbing serum from patient 2-1, but not control serum, with membranes from CaR-transfected HEK293 cells abrogated the stimulation of PTH secretion, as would be expected if anti-CaR antibody had been removed by preabsorption with its antigen (Fig. 9B). Preabsorption of patient or control serum with membranes from nontransfected HEK293 cells had no such effect (not shown).

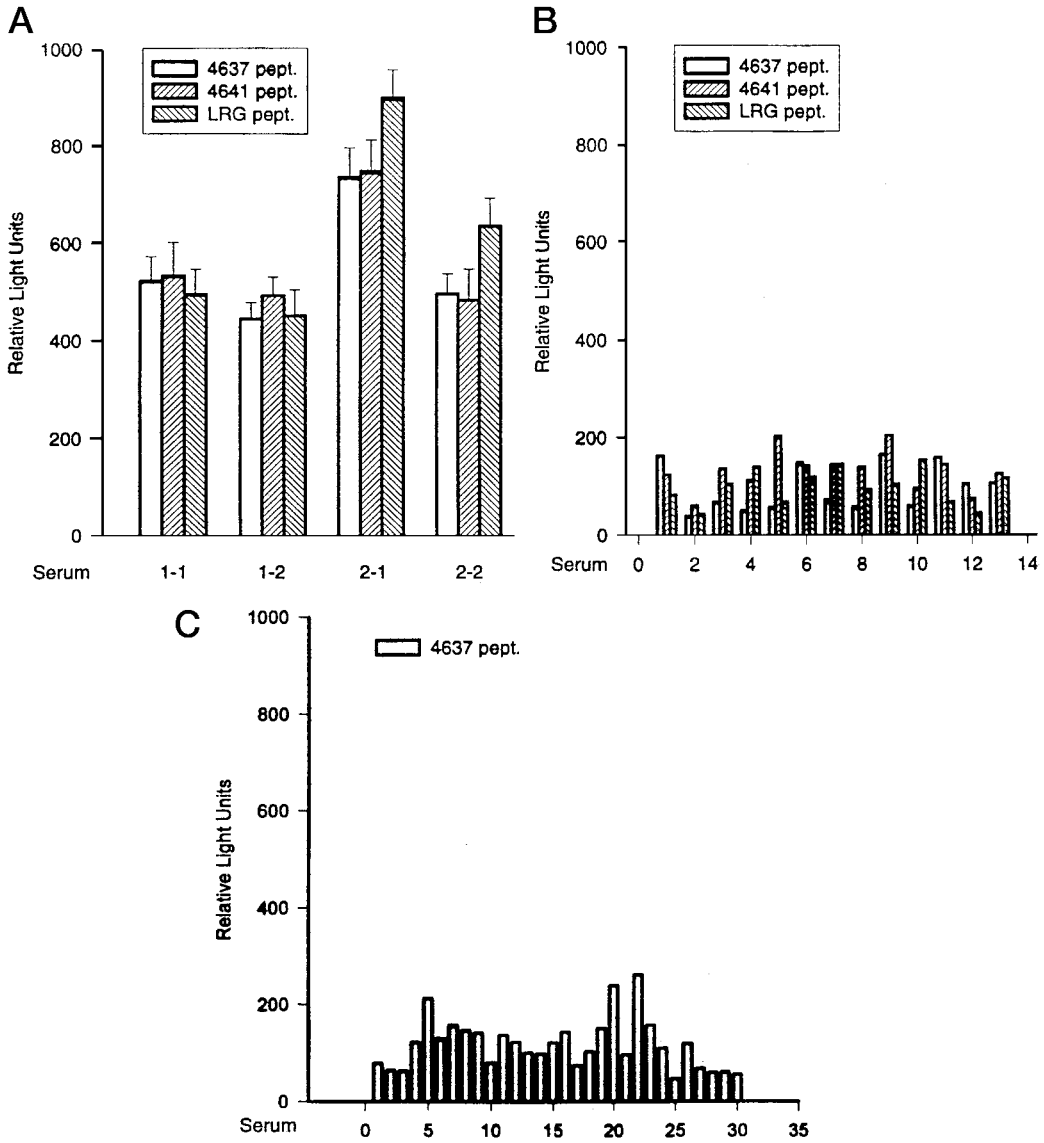

FIG. 5. Binding of protein G-purified IgG from patients and controls to three synthetic peptides derived from CaR's extracellular domain. Ig purified from the sera of the four hypercalcemic patients, 30 normocalcemic controls, or 13 patients with antithyroid antibodies using agarose-bound protein G were tested (at a 1:1000 dilution) for their capacity to bind to peptides corresponding to amino acids 344–358 (denoted 4637 pept; this was the only peptide tested for the normocalcemic controls), 214–236 (denoted 4641 pept), or 374–391 (denoted LRG pept) of the human CaR using an ELISA, as described in *Materials and Methods*. Results for patients (A) and controls (B and C) are expressed as relative light units (compared with the negative controls included in the same experiments) and represent the mean ± SEM. IgG from all four hypercalcemic patients (A) bound to the three peptides to a significantly greater extent than did the control sera from normocalcemic control subjects ($P < 0.05$; B). Binding of the polyclonal or monoclonal anti-CaR antibodies to their respective peptides resulted in activity close to the maximal level measurable in the assay (1000–1100) and was blocked by their respective peptides (not shown). Control experiments with the authentic anti-CaR antibodies also revealed no cross-reactivity of a given anti-CaR antibody with the other peptides (not shown). C, Results of ELISA for serum samples obtained from 13 normocalcemic patients with antithyroid antibodies. Note that the values cluster around 100 relative light units (R.U.; *e.g.* are similar to the normocalcemic control subjects) and that all were at or below 200 R.U., well below the values obtained with the four hypercalcemic patients. As in A, positive controls with the monoclonal or polyclonal anti-peptide CaR antibodies were close to the maximum of 1000–1100 measurable in the assay (not shown).

Lack of inactivating mutations in the CaR gene in patients 1-1 and 2-1

To investigate whether the two families studied here might also have FHH on a genetic basis, we sequenced the CaR genes in the two index cases to search for potentially inactivating mutations. Patient 1 was heterozygous for a previously reported polymorphism in exon 7 in which glycine was changed to arginine (GGG→AGG) in codon 990 (8). Interestingly, the control human DNA purchased from a commercial vendor was homozygous for this polymorphism, which has been reported to be present in up to 15% of normal individuals (8). Otherwise, patient 1-1's CaR gene sequence was completely normal. The sequence of the CaR gene in patient 2-1 was identical to the published sequence of the normal CaR gene.

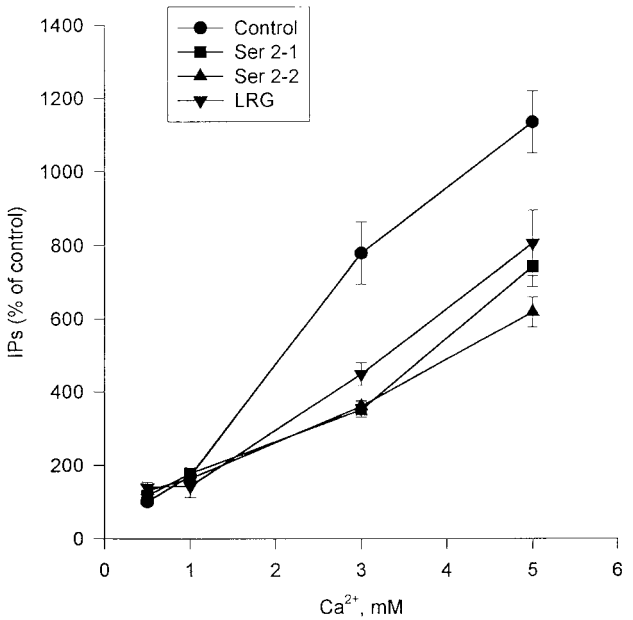

FIG. 6. Autoantibody-mediated inhibition of high Ca^{2+}_o-evoked accumulation of inositol phosphates in HEKCaR cells. HEKCaR cells were cultured overnight with [^{3}H]inositol, washed, and incubated for 30 min at 37 C with 0.5 mM Mg^{2+}_o, 0.2% BSA, 10 mM LiCl, and 0.5, 1.0, 3.0, or 5.0 mM Ca^{2+}_o after preincubation for 10 min at 37 C with affinity-purified serum from patients 2-1 or 2-2 or with control serum purified in like manner from sera pooled from three normocalcemic controls or with a monoclonal antibody raised to peptide LRG (denoted LRG) as described in *Materials and Methods*. The reaction was then terminated with 10% TCA (final wt/vol), and total inositol phosphates were determined as described in *Materials and Methods*. The results indicate the mean ± SEM for three separate experiments, each carried out using duplicate wells. There was statistically significant inhibition of the stimulation of total inositol phosphates (IPs) by 3.0 or 5.0 mM Ca^{2+}_o ($P < 0.05$).

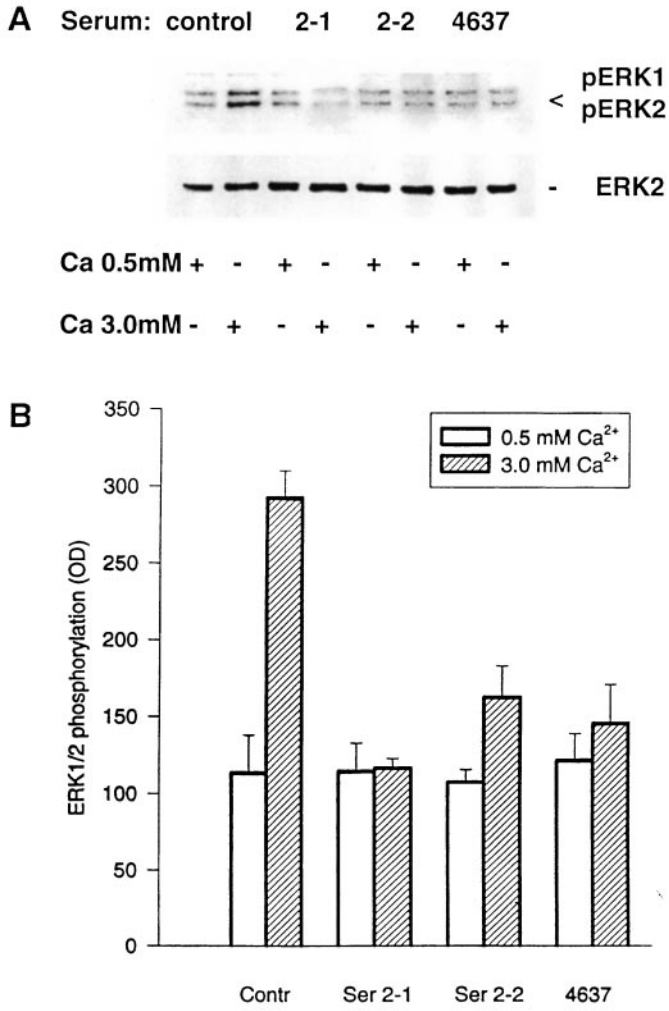

FIG. 7. Autoantibody-mediated inhibition of high Ca^{2+}_o-stimulated ERK1/2 phosphorylation in HEKCaR cells. Serum-deprived HEKCaR cells were incubated for 10 min at 37 C in the presence of 0.5 or 3.0 mM Ca^{2+}_o with affinity-purified serum from patients 2-1 and 2-2, after preincubation for 10 min at 37 C with affinity-purified serum from patients 2-1 and 2-2, or with control serum purified in a similar manner from sera pooled from three normocalcemic controls or with polyclonal affinity-purified, anti-CaR antibody 4637 as described in *Materials and Methods*. A, Results of a representative determination of ERK1/2 phosphorylation (P-ERK1/2) measured using Western blot analysis with a rabbit polyclonal phospho-ERK1/2-specific antiserum and reprobed with a monoclonal anti-ERK2-specific antibody (see *Materials and Methods*; *upper panel*). B, Results (mean ± SEM) pooled from three independent experiments, normalized to those observed at 0.5 mM Ca^{2+}_o with the control level set at 100%. There was statistically significant inhibition by the patients' Ig and by antiserum 4637 of the stimulation of ERK1/2 phosphorylation by 3.0 mM Ca^{2+}_o observed in the presence of Ig from control sera ($P < 0.05$).

Discussion

Circulating autoantibodies occasionally act as receptor agonists or antagonists and mimic genetic disorders arising from activating or inactivating receptor mutations (36–38). Heterozygous inactivating mutations of the CaR gene (10, 39) produce mild to moderate, and occasionally more severe, PTH-dependent hypercalcemia and relative hypocalciuria due to the resultant resistance of the parathyroid glands and kidneys to Ca^{2+}_o, a condition called FHH or familial benign hypercalcemia (3, 4). Conversely, activating CaR mutations, by producing oversensitivity of the same tissues to Ca^{2+}_o, cause hypocalcemia, low normal PTH levels, and relative hypercalciuria (19, 40, 41). We have identified four patients in two families with PTH-dependent hypercalcemia and relative hypocalciuria in three of the four in association with anti-CaR antibodies stimulating PTH release *in vitro*. As with FHH, autoimmune hypocalciuric hypercalcemia results from resistance of parathyroid and kidney to Ca^{2+}_o, although on an acquired, antibody-mediated, rather than a genetic, basis. Anti-CaR antibodies have been described previously in patients with hypoparathyroidism, but they did not modulate the receptor's function in CaR-transfected HEK293 cells (42). To our knowledge, anti-CaR antibodies causing PTH-dependent hypercalcemia have not been described previously. Two cases have been described in which there was lymphocytic infiltration and partial destruction of parathyroid adenomas (43), but in these cases the autoimmune response would be anticipated to reduce, rather than increase, parathyroid function. Furthermore, there was no lymphocytic infiltration of the normal parathyroid glands, suggesting that the immune response was not directed at an antigen present within normal parathyroid cells, such as the CaR.

The two probands did not have identifiable mutations

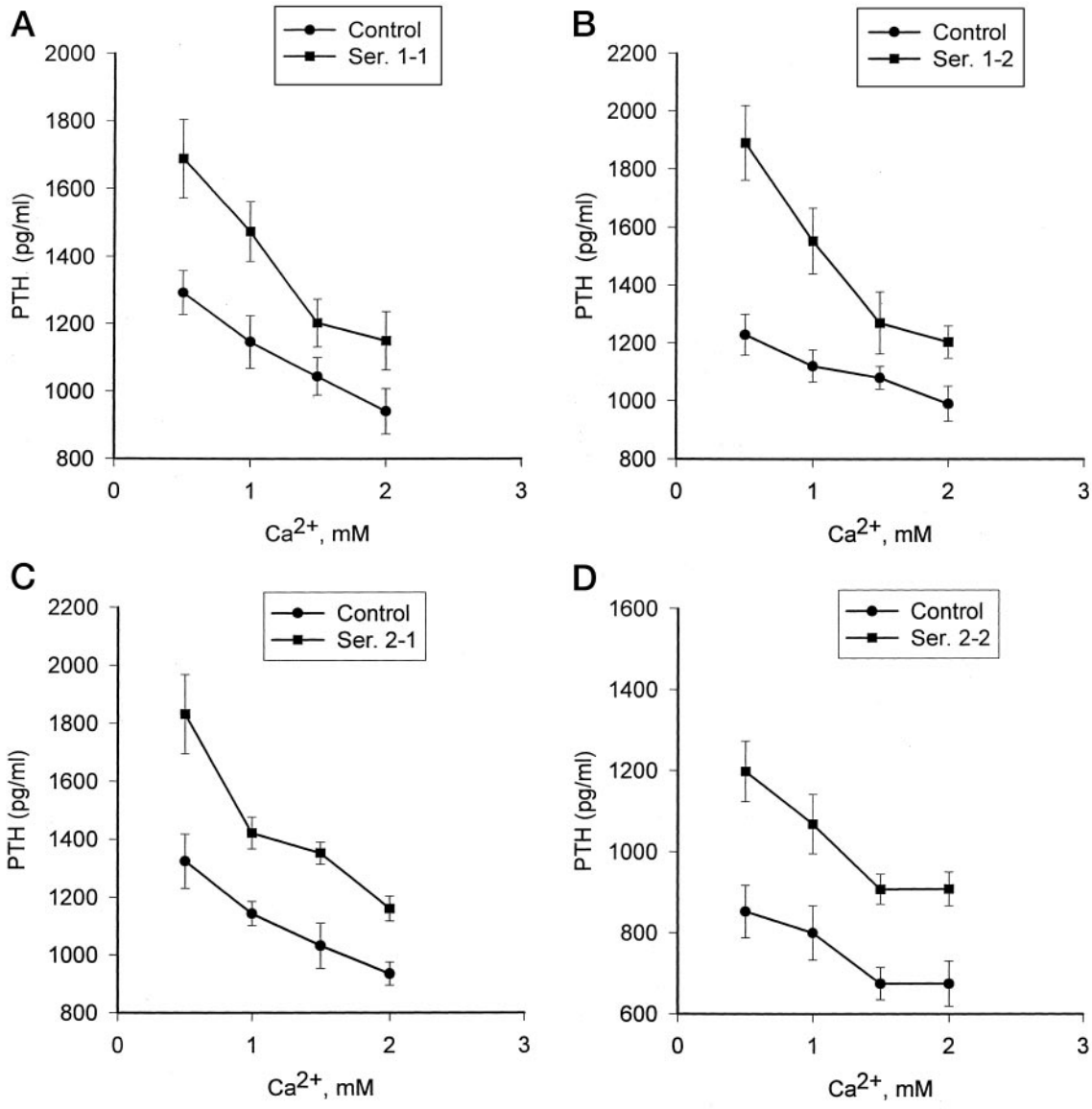

FIG. 8. Ca^{2+}_o-regulated PTH release from human parathyroid cells after preincubation with patient or control sera as indicated in the individual panels. Dispersed human parathyroid cells (1×10^6 cells/0.5 ml) were preincubated with sera from the four hypercalcemic patients or with pooled sera from five normal control subjects and were incubated with varying concentrations of Ca^{2+}_o as described in *Materials and Methods*. Supernatant samples from duplicate incubation vials were collected and assayed for PTH using the whole immunoradiometric assay specific for PTH-(1–84). Results represent the mean ± SEM from three to five experiments using parathyroid cells prepared from different parathyroid glands. PTH release was significantly greater in cells preincubated with sera from the four hypercalcemic patients than in those preincubated with control sera at 0.5, 1.0, 1.5, and 2.0 mM Ca^{2+}_o, except for sera from patients 1-1 and 1-2, in which the stimulation of PTH release did not achieve statistical significance at 1.5 mM Ca^{2+}_o.

within the CaR gene. However, we cannot formally rule out genetic FHH, as approximately one third of patients with FHH do not have mutations identified within the CaR gene. In addition, the syndrome of FHH has been linked in two families to two distinct loci on chromosome 19 (11, 12), although the responsible genes have not yet been identified, precluding mutational analysis. To our knowledge neither the families with linkage of FHH to chromosome 3 nor those with linkage to chromosome 19 have exhibited autoimmunity similar to that in the two families described here.

These patients' sera had anti-CaR antibodies by several criteria: 1) IgG bound to the parathyroid cell surface and colocalized with an authentic anti-CaR antiserum; 2) on Western blots performed using serum affinity-purified using a peptide within the CaR's extracellular domain IgG bound to proteins of molecular weights similar or identical to those recognized by an authentic anti-CaR antiserum raised in a rabbit to the same peptide; and 3) IgG bound to 3 synthetic peptides from the CaR's extracellular domain. In contrast, 13 normocalcemic subjects with antithyroid antibodies and 30 normocalcemic control subjects did not have anti-CaR antibodies or had much lower titers than those of the patients, as assessed by ELISA. It will be of interest in future studies to study more patients with various forms of autoimmunity,

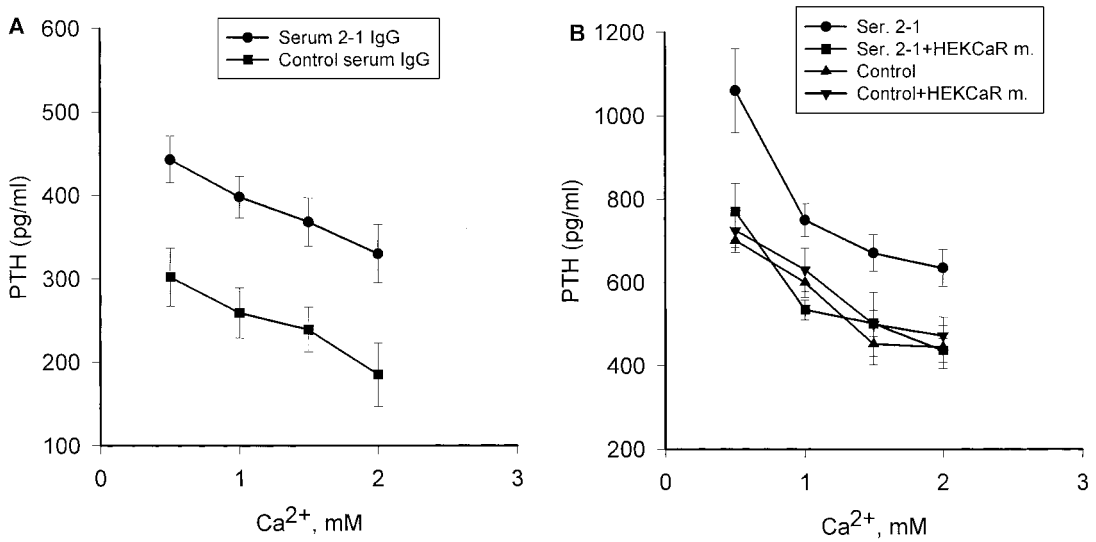

FIG. 9. Purified IgG from patient 2-1 stimulates PTH release, and preabsorption of the serum with membranes from CaR-transfected HEK293 cells blocks its stimulatory effect on PTH release. A, Dispersed parathyroid cells were treated as described in Fig. 7, except that they were preincubated with protein G-purified IgG from the serum of patient 2-1 or from pooled sera from five normal control subjects. Results represent the mean ± SEM from three to five experiments using different parathyroid glands. B, Dispersed parathyroid cells were preincubated with serum samples from patient 2-1 or pooled sera from five normal control subjects that had been preabsorbed with membranes from CaR-transfected HEK293 cells or were preincubated with nonpreabsorbed pooled sera from the control subjects or serum from patient 2-1 as negative and positive controls, respectively, as described in *Materials and Methods*. Ca^{2+}_o-regulated PTH release was determined as explained in Fig. 7. Results represent the mean ± SEM from four or five experiments using different parathyroid glands. PTH release was significantly greater in parathyroid cells pretreated with untreated serum from patient 2-1 than in those pretreated with the same serum preabsorbed with membranes from CaR-transfected HEK293 cells or with untreated control serum at all levels of Ca^{2+}_o ($P < 0.05$), except 1.5 mM Ca^{2+}_o. In the latter case, the difference in PTH release between cells preincubated with untreated serum from patient 2-1 and those preincubated with untreated control serum did not achieve statistical significance. There was no significant difference at any level of Ca^{2+}_o between PTH release from cells pretreated with control serum or control serum preabsorbed with CaR-transfected membranes. There was also no effect of preabsorption of either control serum or that from patient 2-1 with membranes from nontransfected HEK293 cells on Ca^{2+}_o-regulated PTH release (not shown).

including autoimmune hypoparathyroidism (42), to determine the prevalence of anti-CaR antibodies as well as their functional impact, if any, on the CaR.

Sera from all four patients (and purified IgG in the case in which it was studied) stimulated PTH secretion *in vitro*. The abolition of this effect after preincubation of patient 2-1's serum with membranes from CaR-transfected, but not nontransfected, HEK293 cells strongly suggests an effect mediated by anti-CaR antibody. Although we used pathological human parathyroid cells, most such cell preparations retain some CaR expression (23, 33) and some degree of responsiveness to Ca^{2+}_o (35). These results suggest that the anti-CaR antibodies interfere with the receptor's capacity to sense Ca^{2+}_o and, in turn, inhibit PTH secretion. Further evidence in support of this mechanism was provided by the inhibition of CaR-mediated activation of phospholipase C and ERK1/2 MAPK by affinity-purified sera from patients 2-1 and 2-2. Because patients 1-1 and 1-2 had returned to South America before the initiation of these *in vitro* studies, we had insufficient serum samples to perform similar studies using their sera.

Renal actions of the anti-CaR antibodies probably also contributed to our patients' biochemical abnormalities. In FHH an impaired calciuric response to hypercalcemia results from renal resistance to Ca^{2+}_o (3, 4, 44, 45). Therefore, relative hypocalciuria in two of our patients may reflect antibody-mediated, partial inactivation of the CaR in the kidney. It is possible that the fourth patient's (no. 2-2) hypercalciuria resulted from preferential binding of anti-CaR antibodies to the CaR in parathyroid rather than kidney or the presence of additional antibodies with direct or indirect modifying actions on renal calcium handling.

Autoimmune hypocalciuric hypercalcemia could potentially differ clinically from FHH. First, antibody-mediated hypocalciuric hypercalcemia is presumably acquired, rather than being life-long as in FHH, and such acquired hypercalcemia might cause symptoms, unlike the generally asymptomatic nature of FHH. Furthermore, anti-CaR antibodies might not have equivalent access to the CaR in all tissues in which it is expressed (*e.g.* the brain). Thus, symptoms due to effects of hypercalcemia on the brain could be present in patients with autoimmune hypocalciuric hypercalcemia, despite their absence in FHH. The CaR expressed on the luminal aspect of the inner medullary collecting ducts (where the receptor probably impairs urine-concentrating capacity in some hypercalcemic subjects) (46–48) also might not be exposed to anti-CaR antibodies. It is interesting in this regard that patient 1-1, unlike patients with FHH, who concentrate their urine normally (49), complained of polyuria and polydipsia, although urine-concentrating ability was not tested.

It is conceivable that autoimmune hypocalciuric hypercalcemia could occur in a sporadic, rather than a familial, setting and that anti-CaR antibodies might be the sole manifestation of autoimmunity. Thus, this syndrome could be more common than suggested by our studies. Moreover, as

exemplified by patient 2-2, these patients' biochemical features may resemble PHPT more than FHH (*e.g.* PTH-dependent, hypercalciuric hypercalcemia). Based on the admittedly small number of cases studied here, features that might potentially distinguish anti-CaR antibody-mediated hypercalcemia from PHPT may include normal PTH levels and relative or even absolute hypocalciuria in the former. Furthermore, the optimal clinical management of autoimmune hypocalciuric hypercalcemia is far from clear. Anything less than total parathyroidectomy might, as in FHH, fail to definitively cure hypercalcemia, because the anti-CaR antibodies could persist and cause recurrent hypercalcemia, as in patient 2-1. The calcimimetic CaR activators in clinical trials for PHPT might represent a useful therapy for antibody-mediated hypocalciuric hypercalcemia, without the potential toxicity of immunosuppressive agents, because the former reset the elevated set-point of hyperparathyroid glands toward normal (50, 51). A similar resetting of parathyroid and kidney in autoimmune hypocalciuric hypercalcemia might improve or even normalize the associated biochemical abnormalities and symptoms.

In summary, we describe four patients with autoimmune PTH-dependent hypercalcemia due to naturally occurring anti-CaR antibodies. Further studies should reveal how often anti-CaR antibodies produce the biochemical picture of familial or sporadic hypocalciuric hypercalcemia or a clinical presentation more closely resembling primary hyperparathyroidism.

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