

Activating Antibodies to the Calcium-Sensing Receptor in Two Patients with Autoimmune Hypoparathyroidism

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Autoimmune hypoparathyroidism is thought to result from immune-mediated destruction of the parathyroid glands. We encountered two patients with hypoparathyroidism and other autoimmune conditions (Graves' disease and Addison's disease, respectively) in whom autoimmune destruction of the parathyroid glands had not taken place. In the first, a histologically normal parathyroid gland was observed at the time of subtotal thyroidectomy; and in the second, the hypoparathyroidism remitted spontaneously. Both patients had antibodies that reacted with the cell surface of bovine parathyroid cells and human embryonic kidney (HEK293) cells transfected with the extracellular calcium-sensing receptor (CaR) but not with nontransfected HEK293 cells. The antibodies also re-

acted with the same bands on Western analysis of extracts of bovine parathyroid tissue and CaR-transfected HEK293 cells that were identified by an authentic, polyclonal, anti-CaR antiserum and reacted with several peptides with sequences from the CaR's extracellular domain. These anti-CaR antibodies activated the receptor based on their ability to increase inositol phosphate accumulation, activate MAPK, and inhibit PTH secretion. These results, therefore, demonstrate that patients with the biochemical findings of primary hypoparathyroidism can harbor activating antibodies to the CaR, which, in the two cases studied here, did not produce irreversible destruction of the parathyroid glands. (*J Clin Endocrinol Metab* 89: 548–556, 2004)

AUTOIMMUNE HYPOPARATHYROIDISM CAN occur as an isolated clinical abnormality, as part of autoimmune polyendocrinopathy syndrome (APS)-1 or, less commonly, as part of APS-2 (1). APS-1 most commonly comprises mucocutaneous candidiasis, hypoparathyroidism, and Addison's disease. APS-2 includes two or more of the following: Addison's disease, Graves' disease, autoimmune thyroiditis, type 1 diabetes mellitus, primary hypogonadism, myasthenia gravis, or celiac sprue.

Parathyroid histology has been available in relatively few cases of autoimmune hypoparathyroidism and has shown varying degrees of fatty replacement, atrophy, and lymphocytic infiltration (2–7), suggesting the possibility of an autoimmune destructive process. Patients with autoimmune hypoparathyroidism were first demonstrated to have antiparathyroid antibodies in the 1960s (8). Antiparathyroid antibodies have been shown to exert cytotoxic effects on cultured bovine parathyroid cells *in vitro* (9). Patients with adult onset hypoparathyroidism have generalized T cell activation, which could be a manifestation of cell-mediated immunity against parathyroid chief cells, leading to parathyroiditis (10). Thus, both humoral and cell-mediated

immunity could contribute to loss of parathyroid cells in hypoparathyroidism.

The identity/ies of the parathyroid antigens recognized by antiparathyroid antibodies has been obscure until recently. Blizzard *et al.* (8) found that antiparathyroid antibodies reacted with an antigen found in normal parathyroid tissue and in some, but not all, parathyroid adenomas. More recently, Li *et al.* (11) found that 14 of 25 patients (18 with APS1 and seven with hypoparathyroidism and autoimmune hypothyroidism) had antibodies that reacted with the extracellular domain of the extracellular calcium (Ca^{2+}_e)-sensing receptor (CaR). The CaR is the cell surface, G protein-coupled receptor through which parathyroid chief cells, thyroidal C cells, and various kidney cells recognize and respond to changes in Ca^{2+}_e , so as to maintain calcium homeostasis (12). However, there was no apparent functional impact of the antibodies on the CaR in this study, in which sera from several patients with autoantibodies were incubated with CaR-transfected HEK293 cells and changes in the cytosolic calcium concentration (Ca^{2+}_i) were monitored.

We recently encountered two patients whose clinical presentations suggested that they might harbor antibodies to the CaR that activated the receptor. In one, a patient with longstanding hypoparathyroidism and Graves' disease was noted to have a normal parathyroid gland at the time of subtotal thyroidectomy. In the second, a patient with newly diagnosed Addison's disease was also hypocalcemic with an inappropriately normal serum PTH level, indicating the presence of hypoparathyroidism. The hypocalcemia subsequently remitted, however, indicating that there had not been irreversible destruction of the parathyroid glands. In

Abbreviations: APS, Autoimmune polyendocrinopathy syndrome; Ca^{2+}_i , cytosolic calcium concentration; Ca^{2+}_e , extracellular calcium; CaR, calcium-sensing receptor; GF, glomerular filtrate; GFR, glomerular filtration rate; nl., normal; pERK, phospho-ERK; SDS, sodium dodecyl sulfate.

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the studies described herein, both patients were found to harbor antibodies to the CaR that increased the activity of phospholipase C and MAPK and inhibited PTH secretion. Thus, autoimmune hypoparathyroidism can result from autoantibodies to the CaR that activate the receptor, thereby inhibiting PTH secretion, rather than simply exerting a cytotoxic action on the parathyroid cell.

Subjects and Methods

Patient 1

The patient was diagnosed, at the age of 14, with hyperthyroidism attributable to Graves' disease, in Sydney, Australia. Anti-TSH receptor antibodies were positive. He was treated with carbimazole. The same year, he had several seizures and was found to be hypocalcemic. No prior serum calcium measurements were available. At that time, PTH was 0.34 ng/ml [normal (nl.) < 0.4] [340 ng/liter (values in SI units are given in parentheses after the conventional units) (nl. < 400)]. He was treated with vitamin D and calcium supplementation and experienced no further seizures. Over the next 9 yr, antithyroid medication could not be withdrawn without a relapse of the thyrotoxicosis. At that time (23 yr of age), the thyrotoxicosis remained difficult to control because of fluctuating hypo- and hyperthyroidism. At 25 yr of age, he underwent subtotal thyroidectomy. Histological examination was consistent with treated Graves' disease and also revealed a parathyroid gland that was read as normal by three pathologists and showed no lymphocytic infiltration, whereas there were focal accumulations of lymphocytes in the adjacent thyroid tissue. He subsequently required T₄-replacement therapy.

Initial clinical studies were carried out at age 25. Informed written consent was obtained from the patient, and the studies were approved by the institutional ethics committee at the Royal North Shore Hospital in Sydney. Two hundred units of human PTH 1–34 produced a peak cAMP response of 33.2 nmol/100 ml (332 μmol/liter) glomerular filtrate (GF) with a control value of 0.9 nmol/100 ml (9 μmol/liter) GF, and tubular maximum phosphorus (TmPO₄)/GFR fell from 4.1 to 1.8 mg/dl (1.3 to 0.58 mmol/liter), indicating normal PTH sensitivity. Before the study, serum calcium was 7.8 mg/dl, nl. = 8.2–10.2 (1.95 mmol/liter, nl. = 2.1–2.65). At that time, the patient was prescribed 1.5 μg calcitriol daily, as well as supplementation with magnesium and 3 g elemental calcium. Additional laboratory tests were as follows: serum PTH 1–84, 0.39 ng/ml, nl. < 0.4 (390 ng/liter, nl. < 400) [when calcium was 7.8–8.0 mg/dl (1.95–2.0 mmol/liter)]; PTH 1–34, 205 pg/ml, nl. < 130 (205 ng/liter, nl. < 130 ng/liter) [when calcium was 7.8–8.0 mg/dl (1.95–2.0 mmol/liter)]; intact PTH, less than 8 pg/ml, nl. = 10–65 (< 8 ng/liter, nl. = 10–65) [when calcium was 8.96 mg/dl (2.25 mmol/liter)]; PTH 1–84, 0.1 ng/ml, nl. < 0.4 (100 ng/liter, nl. < 400) [when calcium was 9.0 mg/dl (2.25 mmol/liter)]; magnesium, 1.6–2.1 mg/dl, nl. = 1.5–2.6 (0.66–0.86 mmol/liter, nl. = 0.62–1.1); phosphate, 3.6 mg/dl, nl. 2.5–4.7 (1.2 mmol/liter, nl. = 1.2–1.5); creatinine was 0.8 mg/dl, nl. = 0.6–1.2 (70 μmol/liter, nl. = 53–106); 25-hydroxyvitamin D was 29 ng/ml, nl. = 15–53 (72 nmol/liter, nl. 37–130); and 1,25 dihydroxyvitamin D was 43 pg/ml, nl. = 22–62 (107 pmol/liter, nl. = 55–150). A 24-h urine collection, at a time when his serum calcium was 7.8 mg/dl (1.95 mmol/liter), contained 44 mg (1.1 mmol) calcium and 1.0 gm (88 μmol) creatinine. During a 6-h infusion with 400 IU PTH, 1,25 dihydroxyvitamin D increased from 24 to 45 pg/ml (60 to 112 pmol/liter), tubular maximum phosphorus (TmP)/GFR decreased from 4.1 to 1.8 mg/dl (1.32 to 0.58 mmol/liter), and serum calcium increased from 8.0 to 8.2 mg/dl (2.0 to 2.05 mmol/liter). The hypocalcemia, in the presence of normal vitamin D levels and normal or even slightly elevated circulating PTH levels, was not explained.

Patient 2

A 25-yr-old male was admitted on April 21, 2001 to the Brigham and Women's Hospital with 6 months of progressive fatigue, weight loss, vomiting, and salt craving as well as hyperpigmentation of 2-yr duration. Past medical history revealed hypothyroidism, for which he was treated with T₄ (100 μg/d). Family history was positive for celiac disease in a cousin. Physical examination was unremarkable except for hypo-

tension and marked hyperpigmentation. His serum sodium was 124 milliequivalents/liter, nl. = 136–145 (124 mmol/liter); potassium, 5.9 milliequivalents/liter, nl. = 3.5–5 (5.9 mmol/liter); total calcium, 8.0 mg/dl, nl. = 8.8–10.5 (2.0 mmol/liter, nl. = 2.2–2.63); ionized calcium, 1.0 mmol/liter, nl. = 1.13–1.32; albumin, 4.4 mg/dl, nl. = 3.7–5.4 (44 mg/liter, nl. = 37–54); and phosphorus, 4.0 mg/dl, nl. = 2.5–4.5 (1.3 mmol/liter, nl. = 0.8–1.45). Serum magnesium concentration was mildly decreased on three determinations (1.5, 1.6, and 1.7 mg/dl, nl. = 1.8–2.5) (0.62, 0.66, and 0.7 mmol/liter, nl. = 0.74–1.04). Previous serum total calcium concentrations, 8 and 16 months previously, were 9.5 and 9.7 mg/dl (2.38–2.43 mmol/liter), respectively. Intact PTH was 22 pg/ml, nl. = 10–65 (22 ng/liter, nl. = 10–65) when serum total calcium concentration ranged from 7.8–8.5 mg/dl (1.95–2.13 mmol/liter). A repeat PTH was 36 pg/ml (36 ng/liter). 25-Hydroxyvitamin D₃ was 18.5 ng/ml, nl. = 9–43 (22 nmol/liter, nl. = 22–103); 1,25 dihydroxyvitamin D₃, 10.1 pg/ml, nl. = 15–75 (25 pmol/liter, nl. = 37–190); and anti-TPO antibody, 120 IU/ml, nl. = 0–20 IU/ml. ACTH stimulation resulted in serum cortisol levels of 0.2 and 0.5 μg/dl, nl. > 20 (0.006 and 0.015 μmol/liter, nl. > 0.56 at 0 and 60 min), respectively. The patient was treated with fluid resuscitation and stress doses of hydrocortisone (100 mg every 8 h), which were tapered by the time of discharge to 20 mg in the morning and 10 mg at night, with 0.1 μg/d fludrocortisone, 500 mg elemental calcium twice daily, T₄ (100 μg daily) and vitamin D (800 IU/d). One month later, serum total and ionized calcium concentrations were 8.9 mg/dl (2.23 mmol/liter) and 1.19 mmol/liter, respectively. Three months after discharge, 25-hydroxyvitamin D was 26 ng/ml (62 nmol/liter), and 1,25-dihydroxyvitamin D was 39.7 pg/ml (99 pmol/liter). Three and 13 months after discharge, serum total calcium concentrations were 9.2 and 9.7 mg/dl (2.3 and 2.43 mmol/liter), respectively.

The studies of patient 2 at Brigham and Women's Hospital were approved by the Institutional Review Board (IRB).

Methods

Specimen collection and preparation. Serum samples were collected and stored at –20 or –80 C. Some serum samples were heated at 56 C to inactivate complement. There was no difference in the results of experiments carried out using heat-inactivated or non-heat-inactivated serum. Sera were affinity-purified as described below.

Affinity purification of sera and antisera. Sera from the two patients; sera from normocalcemic controls; or rabbit polyclonal, anti-CaR antiserum 4637 were affinity-purified as follows: The affinity columns were coupled to a peptide corresponding to amino acid residues 344–358 of the human CaR, which had been used to raise and affinity-purify rabbit polyclonal antiserum 4637 (13). The sera or antiserum were absorbed on the affinity column in PBS, eluted with 20 mmol/liter HCl (pH 2.5), and immediately neutralized as recommended by the manufacturer. The purified antibodies were stored at –20 C until use.

Immunoperoxidase and immunofluorescence. Cells were fixed in PBS containing 4% formaldehyde at 20 C for 10 min. Immunoperoxidase staining was performed using affinity-purified patient or control sera or affinity-purified, polyclonal anti-CaR antiserum 4637 (13). Bound Igs were detected using peroxidase-conjugated, γ-chain-specific, goat antihuman IgG or goat, antirabbit IgG (Sigma, St. Louis, MO). Two-color immunofluorescence, to detect colocalization of anti-CaR antibodies in sera with polyclonal anti-CaR antiserum 4637, was performed as before (13). Parathyroid cells were incubated with both antiserum 4637 (1:200 dilution) and affinity-purified patient or control serum (1:100 dilution), and then with secondary antibodies [goat antirabbit IgG tagged with Alexa 568 (Molecular Probes, Inc., Eugene, OR) or 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., Hercules, CA) at the Brigham and Women's Hospital Confocal Facility.

Western analysis. Western blot analysis was performed as described (13). After cell lysis, nuclei and cell debris were removed by low-speed centrifugation (1000 × g for 10 min); the resultant cell lysate was used for Western blot analysis. Equal amounts of supernatant proteins were mixed with 2× sodium dodecyl sulfate (SDS)-Laemmli buffer, separated on 7.5% SDS-polyacrylamide gels or on linear 3–9% SDS-polyacrylamide

gradient gels, and transferred to nitrocellulose filters (13). The membranes were then incubated with affinity-purified antiserum 4637 (1:1000) or with affinity-purified patient or control sera, followed by washing and incubation with horseradish peroxidase-conjugated, goat antirabbit or antihuman IgG.

Immunoprecipitation. CaR-transfected HEK293 (HEKCaR) cells were washed with ice-cold PBS and lysed with buffer containing 150 mmol/liter NaCl, 10 mmol/liter Tris-HCl (pH 7.4), 1% glycerol, 1 mmol/liter EGTA, 1 mmol/liter EDTA, 1 mmol/liter sodium *o*-vanadate, a cocktail of protease inhibitors, and 1% Triton X-100 as before (13). After centrifugation at $10,000 \times g$ for 10 min, supernatant protein (500 μ g total lysate) was incubated with affinity-purified sera prepared from the two patients' sera, with control serum pooled from five normocalcemic controls 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, 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 anti-CaR antiserum 4637.

Reactivity of anti-CaR antibodies with synthetic peptides. Immulon II microtiter plates (Dynatech, Chantilly, VA) were incubated overnight at 4 C with 1–5 μ g peptide and washed three times as before (13). Patient or control sera (diluted 1:1000 in PBS-0.05% Tween-20) were then added and incubated at 37 C for 1 h. After washing and incubation overnight at 4 C with 50 μ l/well of peroxidase-conjugated, goat antihuman γ -specific IgG, immunoreactivity was quantified using an ML3000 Microtiter Plate Luminometer (Dynatech).

Preparation of membranes from CaR-transfected and nontransfected HEK293 cells. Confluent monolayers of HEK293 cells, stably transfected with the CaR or nontransfected HEK293 cells, 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 a cocktail of protease inhibitors] as described previously (13). The cells were passed through a 22-gauge needle, 10 times, and nuclei and cellular debris were removed by sedimentation. The supernatants were sedimented at $45,000 \times g$ for 1 h, and the pellets were resuspended in Eagle's MEM containing leupeptin and a calpain inhibitor and saved at -80 C. Resuspended membranes (20 μ g protein) were incubated with 100 μ l patient or control sera in 500 μ l Eagle's MEM for 1 h at 37 C. After the incubations, sera were sedimented, and supernatants were diluted with Eagle's MEM (2% final concentration) and incubated with parathyroid cells (13).

Determination of Ca^{2+}_o -regulated PTH release. Dispersed human parathyroid cells (1×10^6 cells/0.5 ml) were preincubated for 1 h at 37 C with 2% (vol/vol) patient or control sera, washed with Eagle's MEM, and incubated with 0.5 mmol/liter Mg^{2+}_o and varying concentrations of Ca^{2+}_o (0.5–2.0 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 (13). In a second 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). Incubation of the three serum samples from patients 1 and 2 with parathyroid cells did alter trypan blue exclusion, which was 95–100% for cells treated with the patients' sera and sera from normocalcemic controls.

Determination of CaR-stimulated inositol phosphate accumulation. Cells labeled with [3 H]-myo-inositol were incubated with varying concentrations of $CaCl_2$ and 10 mmol/liter LiCl for 30 min after preincubation with affinity-purified patient or control sera (14). The affinity-purified control and patient sera were also included during the incubations after the preincubation. The reactions were terminated with 10% ice-cold trichloroacetic acid (final wt/vol). After centrifugation to remove insoluble debris, trichloroacetic acid was extracted with water-saturated diethyl-ether, and inositol phosphates were separated on Dowex anion exchange columns (Bio-Rad Laboratories, Inc.) as described previously (14).

Determination of CaR-stimulated MAPK activity. For determination of ERK1/2 phosphorylation, monolayers of serum-starved HEKCaR cells were preincubated for 60 min with affinity-purified control serum pooled from five normocalcemic controls, or with affinity-purified sera from patients 1 and 2, and then incubated for 10 min in serum-free medium containing 0.2% BSA with varying concentrations of Ca^{2+}_o as before (13). At the end of the incubation period, the medium was removed, and the cells were washed with ice-cold PBS containing 1 mmol/liter sodium vanadate. The cells were then lysed directly with 100 μ l/well Laemmli sample buffer, the cell lysates were sonicated briefly, and proteins were resolved using SDS-PAGE. After transfer onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH), phospho-ERK (pERK)1/2 were detected by immunoblotting using a rabbit polyclonal antiserum as described previously (13). Quantitative comparisons of phosphorylation of ERK1/2 under various experimental conditions were performed using ImageQuant and a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). Nitrocellulose membranes were then probed using an anti-ERK2 monoclonal antibody (Transduction Laboratories, Inc., Lexington, KY) that detects the protein, independent of its state of phosphorylation, to document equal loading of ERK2 protein.

Statistical analysis. Data represent mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis one way ANOVA on ranks. A *P* value < 0.05 was considered to indicate a statistically significant difference.

Results

Clinical features of the two patients

The clinical and biochemical features of the two patients studied here and their clinical courses are described in detail under *Subjects and Methods*. Both patients had hypocalcemia in the presence of inappropriately normal levels of serum PTH, leading to the diagnosis of hypoparathyroidism. In the second case, the hypoparathyroidism was only transient.

The two patients' sera contain antibodies that bind to parathyroid cells or CaR-transfected HEK293 cells

Figure 1 shows that affinity-purified sera from both patients, but not affinity-purified control serum, bound to the surface of CaR-transfected (but not nontransfected) HEK293 cells, as would be expected if the patients' sera harbored anti-CaR antibodies. The same affinity-purified sera from the two patients, but not that from the controls, bound to the cell surface of bovine parathyroid cells and colocalized with authentic anti-CaR antiserum 4637 (Fig. 1).

The two patients' sera contain anti-CaR antibodies by Western analysis and immunoprecipitation

To provide further evidence that the two patients' sera contained anti-CaR antibodies, we carried out Western analysis using serum samples affinity-purified using the same peptide used to raise polyclonal antiserum 4637 to reduce nonspecific staining resulting from the use of whole serum. These affinity-purified serum samples recognized bands, on Western blots of cell extracts from bovine parathyroid cells, that were identical in size to those recognized by the anti-CaR antiserum 4637 (Fig. 2). The bands corresponded to immature, partially glycosylated monomeric receptor, the fully glycosylated mature form of monomeric receptor, and higher molecular-weight bands consistent with receptor dimers and larger oligomers (15, 16). The two patients' affinity-purified serum samples

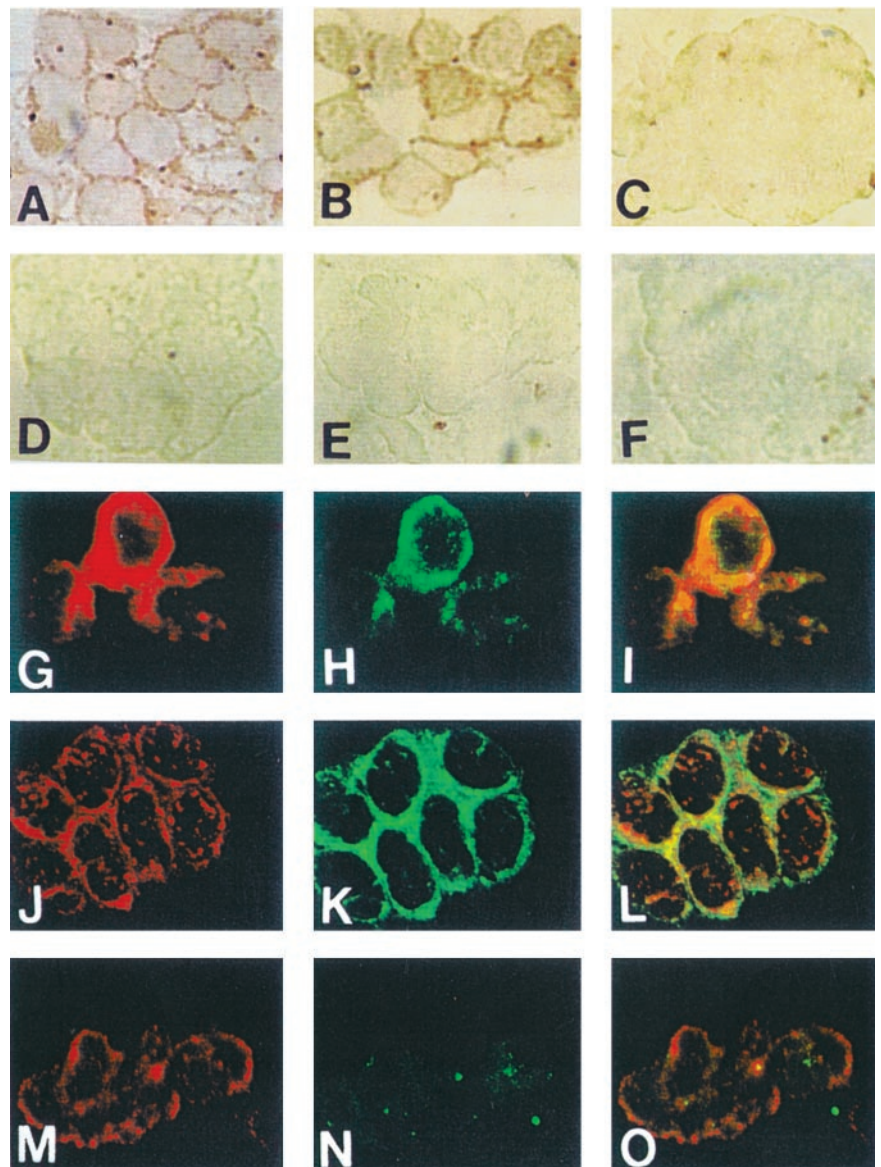


FIG. 1. A–F, Immunoperoxidase staining of CaR-transfected HEK293 cells (A–C) or nontransfected HEK293 (D–F) cells by sera from patients 1 and 2 or by control serum. HEKCaR or HEK293 cells were incubated with the patients' affinity-purified sera or with affinity-purified serum pooled from five normocalcemic controls, then with secondary antiserum (a peroxidase-conjugated, goat antihuman antiserum specific for the γ -chain of IgG), as described in *Methods*. The cell surface of CaR-transfected (but not nontransfected) HEK293 cells was stained by IgGs from the two patients (A and B, and D and E, respectively). The pooled control did not stain either cell type (C and F). Photomicrographs were taken at $\times 630$. G–O, Colocalization of antibodies from patients 1 and 2 (but not control serum) with authentic anti-CaR antiserum 4637 using two color immunofluorescence. Dispersed bovine parathyroid cells were incubated with both antiserum 4637 (1:200 dilution) (red color in G, J, and M) and affinity-purified patient (green color in H and K) or control serum (1:100 dilution) (N), and then with secondary antibodies (see *Methods*). Fluorescence images were collected as described in *Subjects and Methods*. Photomicrographs were taken at $\times 1000$. The yellow color in the merged images indicates colocalization of antiserum 4637 and the anti-CaR antibodies in the two patients (I and L) sera but not in the control sera (O).

showed considerably less immunoreactivity with the CaR expressed in HEKCaR cells relative to antiserum 4637 (Fig. 2). Nevertheless, the patients' sera were able to immunostain CaR-transfected HEK293 cells (e.g. Fig. 1) and to immunoprecipitate the CaR extracted from HEKCaR cells, as demonstrated by subsequent Western blotting with anti-CaR antiserum 4637 (Fig. 2). Again, there are doublets at the positions of receptor monomer and dimer. Immunoprecipitation was also performed with anti-CaR monoclonal antibody LRG as a positive control. This antiserum also immunoprecipitates the receptor, with bands at positions identical to those immunoprecipitated by the patients' affinity-purified sera, when the LRG immunoprecipitate is exposed for a shorter period of time so as to avoid overexposure of the bands (e.g. in Fig. 2, right hand lower panel). As a negative control, affinity-purified serum pooled from five normocalcemic controls was unable to immunoprecipitate CaR-immunoreactive proteins from CaR-transfected HEK293 cells.

The two patients' sera bind to CaR peptides

To demonstrate further that the anti-CaR antibodies in the patients' sera recognized epitopes within the CaR's extracellular aminoterminal, we examined the capacity of sera from both patients to bind to synthetic peptides corresponding to residues 344–358 (4637 peptide), 214–238 (4641 peptide), and 374–391 (LRG peptide) of the human CaR. Figure 3 illustrates that the two patients' purified IgG showed substantially more binding to this synthetic peptide than did purified IgG from normocalcemic subjects that were studied concurrently. We obtained two serum samples on patient 2: one drawn while in the hospital, when his serum total calcium concentration was 1.95–2.13 mmol/liter without any treatment for his hypocalcemia (serum sample 2a); and the second, a month later, when his serum calcium concentration was 2.23 mmol/liter (serum sample 2b), and he was being treated with calcium supplementation. The reactivity of the second sample with the

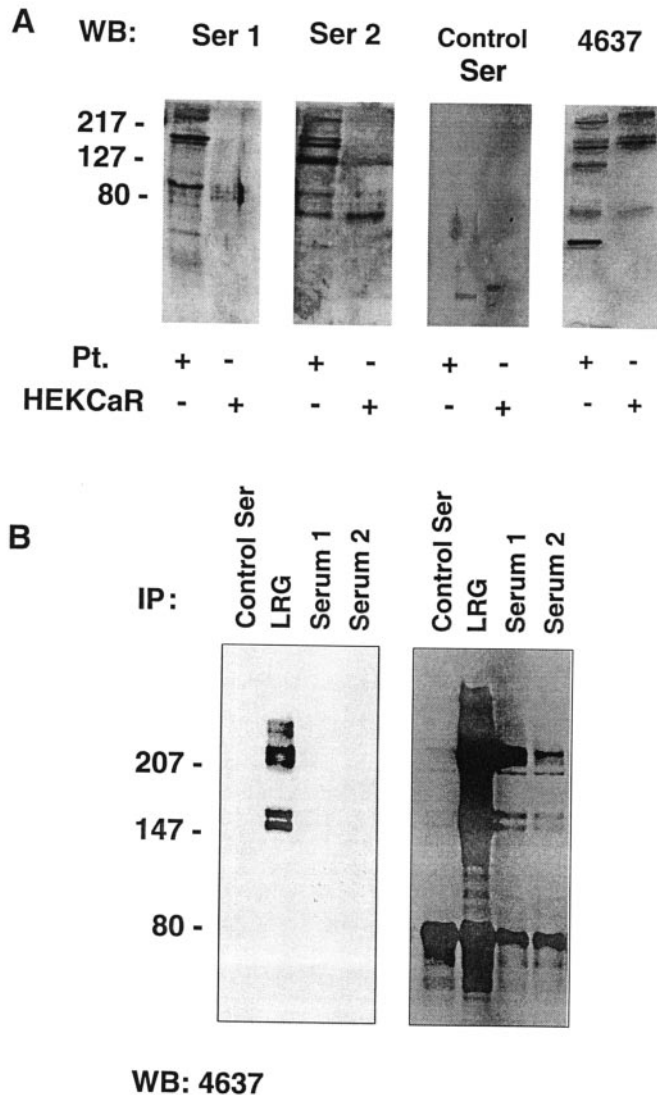


FIG. 2. A, Western blots (WB) of proteins prepared from bovine parathyroid gland (Pt.) or HEKCaR cells using affinity-purified sera from the two patients or control sera or using affinity-purified rabbit anti-CaR antiserum 4637. Protein samples (20 μ g in each lane) were subjected to SDS-PAGE and electrotransfer to nitrocellulose membranes as in *Methods*. After incubation with the affinity-purified sera or antiserum 4637, the blots were washed, and bound antibodies were identified as in *Methods*. Several Western blots were carried out with similar results. B, right-hand panel. Results of Western blotting of proteins immunoprecipitated from lysates of CaR-transfected HEK293 cells by affinity-purified control serum (lane 1), monoclonal anti-CaR antibody LRG (lane 2), or affinity-purified sera from patient 1 (lane 3) and patient 2 (lane 4). After separation of proteins by SDS-PAGE, Western analysis was carried out as in A, using anti-CaR antiserum 4637. The autoradiograms used 5- (*left*) or 30-sec exposures of the same blot. IP, Immunoprecipitation.

CaR peptides was substantially less than that of the first sample and was not statistically significantly different from the control serum.

Stimulation of accumulation of inositol phosphates in CaR-transfected HEK293 cells

To investigate whether the patients' anti-CaR antibodies modulated the function of the receptor, we tested the effects

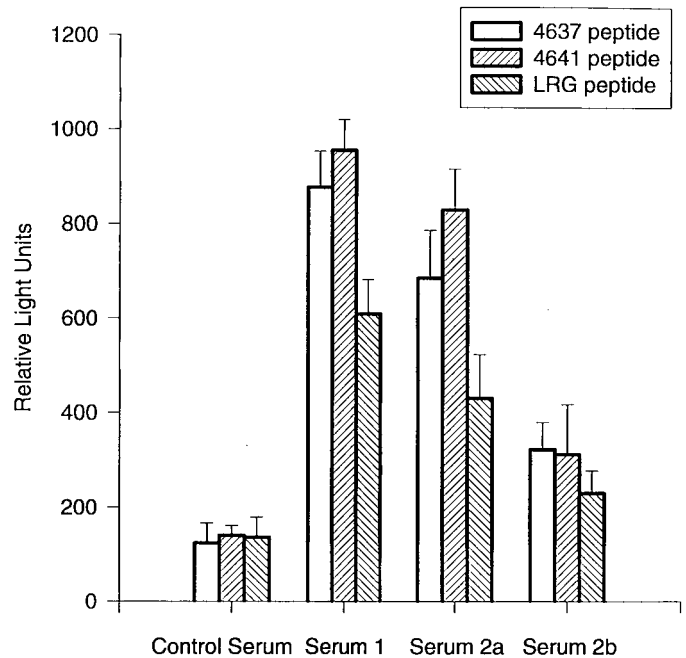


FIG. 3. Binding of sera, from patients 1 and 2 and controls, to synthetic peptides from the CaR's extracellular domain. Sera from the two patients or control serum were tested, at a 1:1000 dilution, for their capacity to bind to peptides corresponding to amino acids 344–358 (4637 peptide), 214–236 (4641 peptide), or 374–391 (LRG peptide) of the human CaR using an enzyme-linked immunosorbent assay (see *Methods*). Results for patients and controls are expressed as relative light units and represent the mean \pm SEM. Two serum samples from patient 2 were tested, denoted 2a and 2b. IgG from both patient 1 and from serum sample 2a bound to the three peptides to a significantly greater extent than did the control sera ($P < 0.05$), whereas the binding of serum sample 2b did not differ from the control.

of affinity-purified sera from the two hypoparathyroid subjects on high Ca^{2+}_o -stimulated accumulation of inositol phosphates in CaR-transfected HEK293 cells, anticipating that the antibodies would activate the CaR. Figure 4 shows that, as expected, the two affinity-purified serum samples increased the accumulation of inositol phosphates at 0.5 and 1.0 mmol/liter Ca^{2+}_o .

Stimulation of MAPK activity in CaR-transfected HEK293 cells

Figure 5 shows that affinity-purified antibodies from patients 1 and 2 stimulated MAPK at 0.5 mmol/liter Ca^{2+}_o , relative to affinity-purified control antibodies. *Panel A* shows an example of a single experiment, whereas the *lower panel* illustrates pooled data from three independent experiments. There was a statistically significant stimulation of MAPK activity by the affinity-purified antibodies from the two patients at 0.5 mmol/liter Ca^{2+}_o .

Patients' sera inhibit PTH release

Because it was not feasible to obtain normal human parathyroid cells, we used parathyroid cells obtained from parathyroid adenomas or cases of primary parathyroid hyperplasia to test the effects of the patients' sera on Ca^{2+}_o -

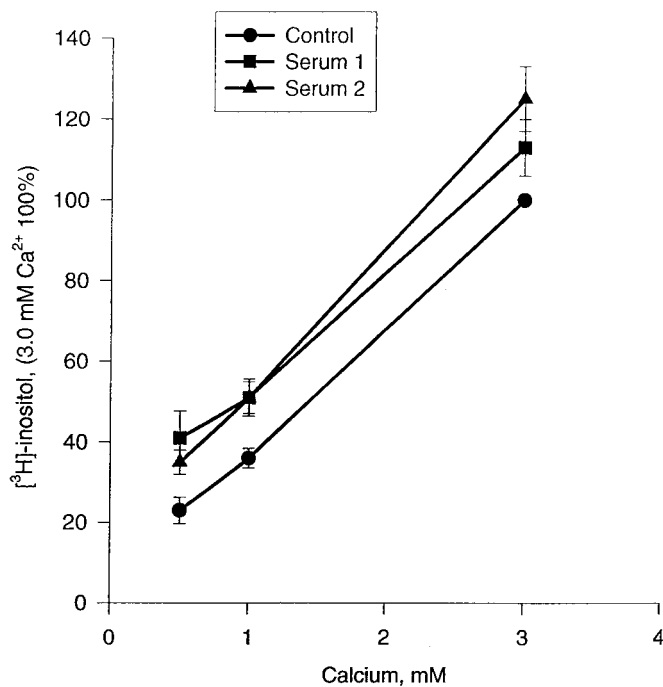


FIG. 4. Autoantibody-mediated stimulation of high Ca^{2+} -evoked accumulation of inositol phosphates in HEKCaR cells. HEKCaR cells were cultured overnight with [^3H]-inositol, washed, and incubated for 30 min at 37 C with 0.5 mmol/liter Mg^{2+} , 0.2% BSA, 10 mmol/liter LiCl, and 0.5, 1, or 3 mM Ca^{2+} after preincubation for 10 min at 37 C with affinity-purified sera from patients 1 and 2 or with affinity-purified control sera. Total inositol phosphates were then determined as in *Methods*. Results indicate the mean \pm SEM ($n = 4-6$). There was a statistically significant stimulation of total inositol phosphates at 0.5 and 1.0 mM Ca^{2+} , relative to that seen with affinity-purified control sera ($P < 0.05$).

regulated PTH release. Pathological parathyroid glands from patients with primary hyperparathyroidism, although expressing lower levels of the CaR than normal bovine or human parathyroid glands, usually retain some degree of responsiveness to Ca^{2+} . Figure 6 illustrates that the serum samples from the two patients inhibited PTH release substantially (both at low and at higher Ca^{2+}). In the case of patient 2, there were two serum samples (one at the time when the patient was overtly hypocalcemic, and the second at a time when his serum calcium concentration was just above the lower limit of normal while being treated with calcium supplementation). Both serum samples produced comparable degrees of inhibition of Ca^{2+} -regulated PTH release.

Preabsorption of serum from patient 2 (there was insufficient serum from patient 1 to perform the same study) with membranes prepared from CaR-transfected (but not non-transfected) HEK293 cells partially reversed the inhibitory effect of the serum on PTH release (Fig. 7).

Discussion

We have identified two patients with hypoparathyroidism who have autoantibodies to the CaR by several criteria. First, both patients' sera had IgG that bound to bovine parathyroid cells and colocalized on the cell surface with an authentic

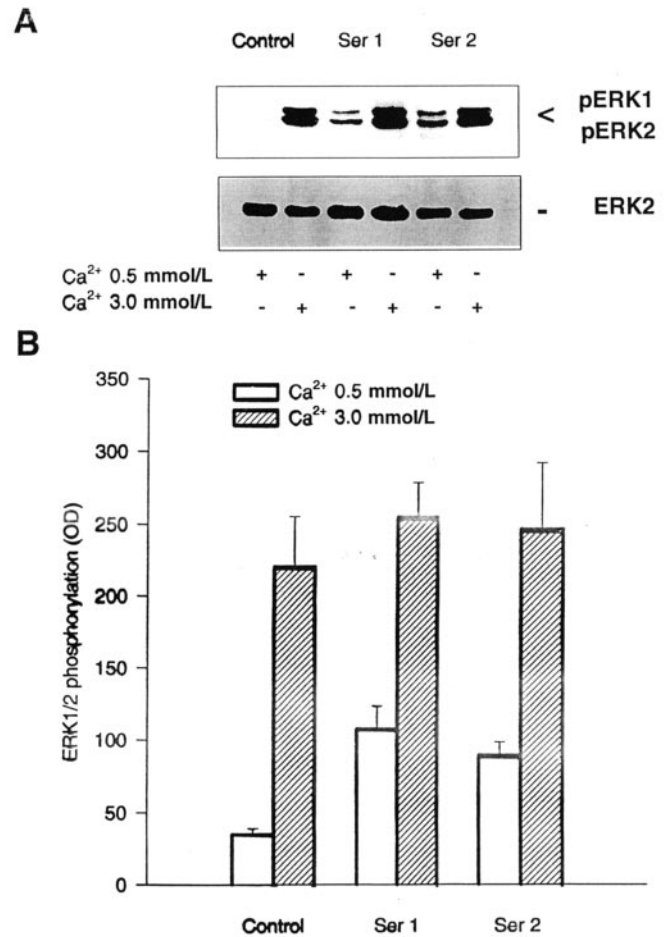


FIG. 5. Autoantibody-mediated stimulation of ERK1/2 phosphorylation in HEKCaR cells. Serum-deprived HEKCaR cells were preincubated for 30 min with affinity-purified serum from patient 1 or 2, or with affinity-purified control serum as in *Methods*. They were then incubated with the same serum samples for 10 min at 37 C in the presence of 0.5 or 3.0 mmol/liter Ca^{2+} . A, Representative experiment performed using Western blotting with an antibody specific for phospho-ERK1/2. The blot was then stripped and reprobbed with a monoclonal, anti-ERK2-specific antibody to document equal loading of the lanes. B, Results (mean \pm SEM) pooled from three independent experiments, expressed in arbitrary units of OD. There was a statistically significant stimulation of ERK1/2 phosphorylation at 0.5 mmol/liter Ca^{2+} by the patients' sera, relative to that observed with the control serum ($P < 0.05$).

anti-CaR antiserum. Second, both patients' sera harbored antibodies that reacted with the cell surface of CaR-transfected HEK293 cells but not with nontransfected HEK293 cells, which do not express the receptor endogenously. The difference in immunoreactivity of the sera with CaR-transfected *vs.* nontransfected HEK293 cells strongly suggests that the patients' antibodies bound to the CaR.

Third, purified IgG from the patients' sera, when used for Western analysis on extracts of bovine parathyroid glands, yielded results nearly identical to those observed with an authentic anti-CaR antiserum. As with the anti-CaR antiserum, the patients' autoantibodies recognized both CaR monomers and dimers, including the characteristic immunoreactive doublet that arises from the presence of both the

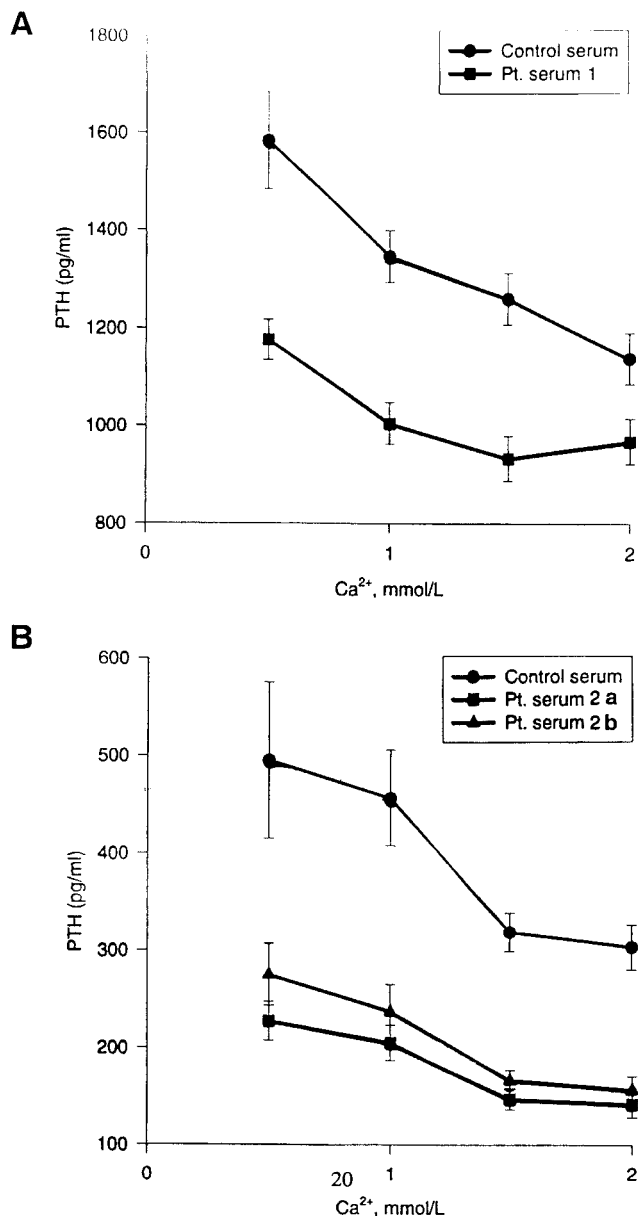


FIG. 6. Ca^{2+} -regulated PTH release from human parathyroid cells after preincubation with patients or control sera. Dispersed human parathyroid cells ($1 \times 10^6/0.5$ ml) were preincubated with 2% serum from the two patients (A, patient 1; B, patient 2) or with control serum and were then incubated with the same sera and varying concentrations of Ca^{2+} , as in *Methods*. Supernatant samples from duplicate incubation vials were assayed for PTH using the whole immunoradiometric assay specific for PTH (1–84) as in *Methods*. Results indicate the mean \pm SEM ($n = 6$ –10). Note that different parathyroid glands were used for A and B, resulting in different values for PTH release and different suppressibility by high Ca^{2+} . In A, PTH release was significantly lower in cells preincubated with the serum from patient 1 than with control serum at 0.5, 1.0, 1.5, and 2.0 mmol/liter Ca^{2+} ($P < 0.05$). Two serum samples from patient 2 were tested, yielding comparable results (denoted 2A and 2B). In B, PTH release was significantly lower in cells preincubated with serum a or serum b from patient 2 than in those preincubated with control serum at 0.5, 1.0, 1.5, and 2.0 mmol/liter Ca^{2+} ($P < 0.05$) (serum a) or 1.0, 1.5, and 2.0 mmol/liter ($P < 0.05$) (serum b). Trypan blue exclusion in cells incubated with the two patients' sera (95–100%) did not differ from that of cells incubated with the control sera that had been preabsorbed with membranes from nontransfected HEK293 cells.

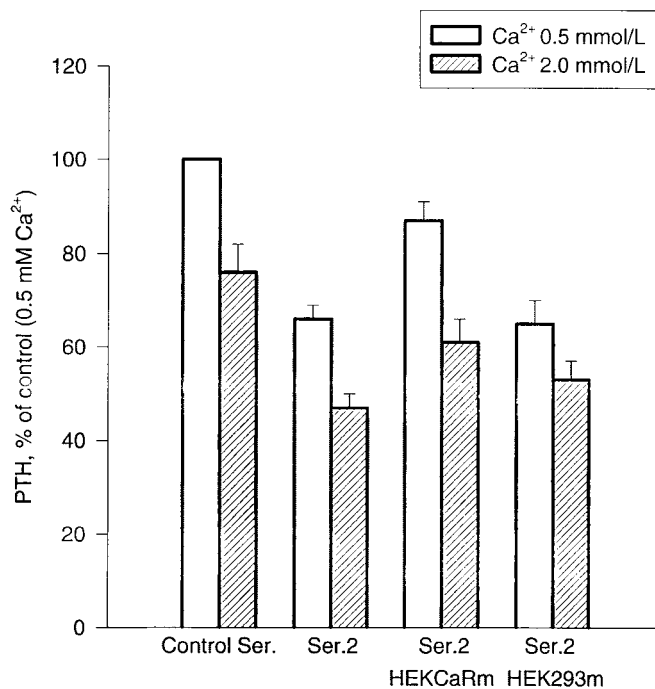


FIG. 7. Effect of preabsorption of anti-CaR antibodies from patient 2 with membranes from HEKCaR or HEK293 cells on Ca^{2+} -regulated PTH release. Dispersed human parathyroid cells were preincubated with 2% serum from patient 2 or 2% serum pooled from five normal control subjects as positive (Ser 2) and negative controls (Control Ser), respectively, or were preincubated with serum preabsorbed with membranes from HEKCaR cells (Ser 2 HEKCaRm) or nontransfected HEK293 cells (Ser 2 HEK293m). The cells were then incubated with the same sera as during the preincubation for 1 h at 37 C, and PTH release was determined in supernatant samples as described in *Subjects and Methods*. There was significant inhibition of PTH release by the serum from patient 2 that had not been preabsorbed, relative to the cells preincubated with the nonpreabsorbed control serum ($P < 0.05$). There was no significant difference between the cells preincubated with nonpreabsorbed serum from patient 2 and those preincubated with serum preabsorbed with membranes from non-CaR-transfected HEK293 cells. Preincubation with serum preabsorbed with membranes from CaR-transfected HEKCaR cells, in contrast, resulted in PTH release that was significantly greater at 0.5 mmol/liter Ca^{2+} than with cells preincubated with nonpreabsorbed serum from patient 2 or cells preincubated with serum from patient 2.

immature, high mannose- and the fully mature, glycosylated forms of the receptor (16). Of interest, the patients' sera recognized the CaR in parathyroid extracts considerably better than in extracts of HEKCaR cells. The patients' sera clearly do react with the nondenatured CaR in HEKCaR cells, however, because they immunostained the receptor on transfected (but not on nontransfected) cells and were able to immunoprecipitate the receptor from the CaR-transfected HEK cells. Fourth, purified IgG from both patients' sera reacted with several peptides corresponding to sequences within the CaR's extracellular domain. Taken together, these data document that both patients harbored anti-CaR antibodies.

It should be pointed out that, because we did not sequence these two patients' CaR genes, we cannot formally exclude the possibility that they had activating mutations of the CaR. Such a mutation would be very unlikely in patient 2, in whom hypocalcemia was only transient. Regardless, how-

ever, they both had antibodies to the CaR that activated the receptor.

In addition to reacting with the CaR on the cell surface of parathyroid and CaR-transfected cells, these anti-CaR antibodies modulated three aspects of the function of these two cell types in ways that provide strong evidence that they activate the receptor. First, affinity-purified antibodies from both patients stimulated the accumulation of total inositol phosphates in CaR-transfected HEK293 cells at 0.5 and 1 mmol/liter Ca^{2+}_o , an index of activation of phospholipase C. Second, affinity-purified antibodies from both patients stimulated MAPK activity at 0.5 mmol/liter, another established parameter of activation of the CaR in a variety of cell types (17–20). Third, serum samples from the two subjects inhibited PTH release from dispersed human parathyroid cells. Although the reduced PTH secretion could have conceivably resulted from nonspecific damage to the parathyroid cells, such an effect would have been expected to reduce rather than increase CaR-stimulated accumulation of inositol phosphates and activation of MAPK.

It is possible that patient 1's anti-CaR antibodies exerted greater effects on the parathyroid than on the kidney. That is, at a time when he was mildly hypocalcemic, his urinary calcium excretion was only 1.1 mmol/24 h. If the anti-CaR antibodies activated the CaR in the kidney, one might have expected the relative or absolute hypercalciuria that is observed in patients with activating mutations of the CaR rather than the overt hypocalciuria seen in this patient. Any reasons why there might be such differential reactivity of the anti-CaR antibodies with parathyroid and kidney must remain conjectural for the moment.

Patient 2 had two serum samples, one drawn at a time when his hypoparathyroidism was first diagnosed; and the second, a month later, when he was being treated with calcium supplementation and his serum calcium concentration was just above the lower limit of normal. Subsequently, the patients' serum calcium concentration totally normalized, returning to levels in the mid-normal range, as it had been 8 and 16 months before the diagnosis of his Addison's disease. It is of interest that his second serum sample, despite the fact that its activity had decreased by more than 50% as assessed by ELISA, still inhibited PTH release to an extent similar to the first sample. It should be pointed out in this regard, however, that the dilution of serum used for the ELISA was 1:1000, whereas that used for studying Ca^{2+}_o -regulated PTH release was 1:50. Therefore, the more concentrated serum used for studying secretion may still have exerted a biological effect that was not observed with the 20-fold-lower concentration used in the ELISA. It is also difficult to know how to compare the relative concentrations of the anti-CaR antibody to which parathyroid cells would be exposed *in vivo* and *in vitro*. It would clearly be of interest to obtain a follow-up serum sample, now that this patient's serum calcium has completely normalized, to assess whether or not the antibody was still present and exerted effects on CaR-mediated PTH release, MAPK activity, and the accumulation of inositol phosphates.

It should be pointed out that this patient had hypomagnesemia during the time that he was hypoparathyroid. It is possible that the hypomagnesemia, albeit mild, contributed

to the genesis of the hypocalcemia (21). Conversely, it is possible that the hypomagnesemia was the result, rather than the cause, of the patient's hypoparathyroidism. For instance, it is possible that binding of the anti-CaR antibodies to the receptor in the distal tubule would result in wasting of both calcium and magnesium. Of interest in this regard, some patients with activating mutations of the CaR exhibit hypomagnesemia (22). Regardless of the relationship of this patient's hypomagnesemia to his hypocalcemia, however, he had anti-CaR antibodies by several criteria, which inhibited PTH secretion *in vitro*, even if we cannot formally prove that they caused the hypocalcemia *in vivo*.

Others have shown previously that patients with autoimmune hypoparathyroidism can harbor anti-CaR antibodies. Li *et al.* (11) demonstrated that 14 out of 25 patients with autoimmune hypoparathyroidism had anti-CaR antibodies in their serum. However, these authors were unable to document any functional impact of these sera on the Ca^{2+}_i . We believe that this latter result was most likely observed because the binding of the antibodies to the CaR *in vitro* was too slow to produce the characteristic transient increase in Ca^{2+}_i evoked by activation of the receptor by high Ca^{2+}_o . The transient high Ca^{2+}_o -elicited elevation in Ca^{2+}_i results from activation of phospholipase C by the CaR, with subsequent production of inositol trisphosphate (IP_3) and release of calcium from intracellular stores. Our assay, in contrast, was based on a more direct measurement of PLC activity; namely, quantitation of the high Ca^{2+}_o -evoked production of cellular inositol phosphates.

Posillico *et al.* (23) showed that antibodies to the parathyroid cell surface were present in eight of 23 cases of idiopathic hypoparathyroidism. In three of these cases, the patients' sera inhibited PTH secretion by about 50% at both low (0.5 mmol/liter) as well as higher (1.5 mmol/liter) levels of Ca^{2+}_o . Moreover, in one of the three cases, the titer of anti-parathyroid antibody and its ability to inhibit PTH secretion *in vitro* decreased progressively during a period of time when the patient's hypoparathyroidism spontaneously improved. In retrospect, it is possible that these three cases harbored activating, anti-CaR antibodies. Of interest, the three patients described by Posillico *et al.* and the two patients presented here all had adult-onset hypoparathyroidism. Further studies are needed to determine whether activating anti-CaR antibodies are present in patients with APS-1 and childhood onset of hypoparathyroidism.

It would be of interest to determine whether, under appropriate experimental conditions, the sera from the two patients described here could exert cytotoxic effects. Brandi *et al.* (9) demonstrated the presence of cytotoxic antibodies that reacted with bovine parathyroid cells in patients with autoimmune hypoparathyroidism. In any event, both of our patients had evidence that the parathyroid glands had not been damaged by immune mechanisms. In the first case, a histologically normal parathyroid gland was obtained at the time of subtotal thyroidectomy, 12 yr after hypoparathyroidism was first diagnosed. In the second case, hypoparathyroidism remitted spontaneously.

The presence of anti-CaR antibodies that exert functional actions without compromising cellular viability has diagnostic (as well as therapeutic) implications. Because the para-

thyroid glands would be intact in such cases, they would presumably retain the capacity to respond to a reduction in Ca^{2+} , with increased PTH secretion. As a diagnostic test, administration of a calcilytic could potentially be sensed by functional parathyroid tissue, even in the presence of an activating antibody, as a reduction in Ca^{2+} , thereby eliciting increased PTH secretion. Furthermore, just as administration of a calcilytic elevates Ca^{2+} from a normal to an elevated level in the euparathyroid state, it might be possible to restore normocalcemia in a hypoparathyroid patient with an activating antibody if the calcilytic were able to antagonize the effect of the antibody (24).

In summary, we describe two patients with activating antibodies to the CaR, who exhibited hypocalcemia and the clinical and biochemical picture of hypoparathyroidism. Additional studies are needed to determine the prevalence of activating, anti-CaR antibodies in patients with autoimmune hypoparathyroidism in the setting of idiopathic hypoparathyroidism, APS-1, and adult onset hypoparathyroidism with other endocrine deficiencies.

Acknowledgments

Polyclonal rabbit anti-CaR antisera 4641 (raised against amino acids 214–238 in the human CaR, referred to as 4641 peptide) and 4637 (raised against amino acids 344–358 in the human CaR, referred to as 4637 peptide), as well as monoclonal anti-CaR antibody LRG (raised against amino acids 374–391 in the human CaR, referred to as LRG peptide), were gifts from Dr. Karen Krapcho and Edward Nemeth (NPS Pharmaceuticals, Inc., Salt Lake City, UT) and Drs. Allen Spiegel and Paul Goldsmith [National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIH), Bethesda, MD], respectively.

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