The Calcium-sensing Receptor Is Localized in Caveolin-rich Plasma Membrane Domains of Bovine Parathyroid Cells*

(Received for publication, March 2, 1998, and in revised form, June 11, 1998)

Olga Kifor§, Ruben Diaz‡, Robert Butters‡, Imre Kifor‡, and Edward M. Brown‡

From the §Endocrine-Hypertension Division and Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts 02115 and the ¶Endocrine Division, Children’s Hospital, Boston, Massachusetts 02115

Parathyroid cells have an intracellular machinery for parathyroid hormone (PTH) secretion that is inversely regulated by the extracellular calcium concentration (Ca2+). The recently characterized Ca2+-sensing receptor (CaR) is a G protein-coupled, seven-transmembrane receptor mediating the inhibitory effects of high Ca2+ on PTH secretion. The CaR’s precise cell surface localization and the signal transduction pathway(s) mediating its inhibitory effects on PTH secretion have not been characterized fully. Here, we demonstrate that the CaR resides within caveolin-rich membrane domains in bovine parathyroid cells. Chief cells within bovine parathyroid glands exhibit a similar pattern of staining for caveolin-1 and for alkaline phosphatase, a glucosylphosphatidylinositol-anchored protein often enriched in caveolae. Purified caveolin-enriched membrane fractions (CEMF) from bovine parathyroid cells are highly enriched in the CaR and alkaline phosphatase. Other signaling proteins, including Gα11, eNOS, and several protein kinase C isoforms (i.e. α, δ, and ω), are also present in CEMF. Activation of the CaR by high Ca2+ increases tyrosine phosphorylation of caveolin-1 in CEMF, suggesting that CaR-mediated signal transduction potentially involved in Ca2+-regulated processes in parathyroid cells occur in caveolae-like domains.

* This work was supported by United States Public Health Service Grants DK44588 and DK52005 (to O. K. and E. M. B.), The St. Giles Endowment Fund, and the Boston Pediatric Endocrinology Society Genentech Scholar Award, and United States Public Health Service Grant DK02530 (to R. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Endocrine-Hypertension Division, Brigham and Women’s Hospital, 221 Longwood Ave., Boston, MA 02115.
‡ Address correspondence to: Robert Butters, Robert Butters, Endocrine Division, Children’s Hospital, Boston, Massachusetts 02115.
§This text was published by the American Society for Biochemistry and Molecular Biology, Inc.
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
Ca2+ protein kinase C isoforms (5-bromo-4-chloro-3-indolyl phosphate tric-oxide synthase; PAGE, polyacrylamide gel electrophoresis; BCIP, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt; NBT, nitroblue tetrazolium chloride; PBS, phosphate-buffered saline; PM, plasma membranes; PNS, postnuclear supernatant; GPCR, G protein-coupled receptor; AP, alkaline phosphatase; GPI, glycosyl-phosphatidylinositol; Ca2+-o, extracellular calcium; CEMF, caveolin enriched membrane fractions.

Ca2+ ions are essential for numerous biological functions, including both vital extracellular processes (i.e. blood clotting, intercellular adhesion, and skeletal integrity) as well as intracellular processes (e.g. regulation of hormonal secretion, cell division, and motility) (1). The parathyroid glands play a central role in maintaining near constancy of the extracellular calcium concentration (Ca2+) by sensing small changes in Ca2+ (1–3). Parathyroid hormone (PTH) secretion is reduced at high Ca2+, a process mediated by a recently characterized, cell surface Ca2+-sensing receptor (CaR) (4, 5). The CaR is a G protein-coupled, seven-transmembrane receptor that is also a key mediator of direct effects of Ca2+ on kidney and several other cells (4–7). It plays an important role not only in normal calcium homeostasis but also in abnormal states of mineral ion metabolism (8–11). Several lines of evidence suggest that the CaR is crucial for Ca2+-regulated PTH secretion. (i) The pharmacological profile for CaR agonist-induced inhibition of PTH secretion is essentially identical to that for the cloned CaR (1, 4). (ii) Inactivating CaR mutations in the heterozygous and homozygous states causes mild and severe increases in PTH secretion, respectively, in the hypercalcemic disorders, familial hypocalciuric hypercalcemia, and neonatal severe hyperparathyroidism (1, 8, 9). Conversely, activating mutations produce a form of autosomal dominant hypocalcemia with inappropriate low serum PTH levels (8, 9). (iii) Hyperplastic and adenomatous parathyroid glands frequently exhibit an increased set-point for PTH secretion (the level of Ca2+ half-maximally inhibiting hormonal release). CaR immunostaining in parathyroid adenomas and in hyperplastic parathyroid glands from patients with severe uremic hyperparathyroidism is reduced relative to that in normal parathyroid glands (10, 11). (iv) The CaR agonist, R-568, effectively inhibits PTH secretion and reduces Ca2+ in hyperparathyroid patients (12). (v) The loss of high Ca2+-evoked inhibition of PTH secretion in cultured bovine parathyroid cells correlates closely with the concomitant decreases in CaR mRNA and protein expression (13).

The CaR couples to several signal transduction pathways. In parathyroid cells, the activated CaR stimulates phospholipase C with subsequent increases of inositol 1,4,5-trisphosphate, 1,2-sn-diacyl-glycerol, and the cytosolic calcium concentration (Ca2+1,3) (2, 3, 14). High Ca2+-evoked phospholipase C stimulation involves a pertussis toxin-insensitive G-protein, probably Gα11, both of which are expressed in bovine parathyroid (15). Two other phospholipases, phospholipases A2 and D, are also CaR-activated (14). Thus, the CaR controls multiple signaling pathways (1, 4, 14). The signal transduction pathway(s) underlying CaR-mediated inhibition of PTH secretion has not been well characterized. Inhibition of PTH release correlates with rises in Ca2+1, a known stimulator of secretion in other cells. Changes in Ca2+ eAMP, and arachidonic acid metabolites have been invoked in CaR-mediated inhibition of PTH secretion (1–3, 14); however, their relative importance has not been well established.

Parathyroid chief cells contain alkaline phosphatase (AP) within their apicolateral domains (16, 17) that is anchored to the outer leaflet of the plasma membrane by a glucosylphosphatidylinositol (GPI) anchor.2 GPI-anchored AP is clustered within caveolae in several different cell types (18, 19). Caveolae
are plasmalemmal microdomains where multiple signaling molecules are concentrated (18–21) and may be similar to the protein-containing clusters of glycosphingolipids and cholesterol, which Simons and Ikonen have described as mobile rafts within the membrane bilayer (22, 23). A major protein component of caveolae are caveolins, integral membrane proteins thought to play a key role in their structural organization (24, 25). Caveolae may be involved in transcytosis, potocytosis, and signal transduction (18–21). Growth factor receptors (e.g. for epidermal growth factor and platelet-derived growth factor) and G protein-coupled receptors (i.e. the endothelin, bradykinin, and muscarinic acetylcholine receptors) can reside in caveolae (26–30). Caveolae can also contain other signaling components, such as heterotrimeric G proteins, adenylate cyclase, Ras/Raf, and protein kinase C (PKC) isoforms (31–35). Endothelial nitric-oxide synthase (eNOS) is markedly enriched in caveolae (36, 37), and interaction between eNOS and caveolin may be regulated by Ca$^{2+}$ and calmodulin (37). Caveolin was originally identified as a major v-src substrate in Rous sarcoma virus-transformed cells (20, 39), which is constitutively phosphorylated on tyrosine (38–40). Caveolin tyrosine phosphorylation also occurs in normal cells but in a tightly regulated fashion (39, 40). Caveolae may also be involved in Ca$^{2+}$-mediated signal recognition and transduction in parathyroid cells, potentially including the poorly understood in-caveolin-enriched fractions. Thus, our results suggest that CaR-mediated signal recognition and transduction in parathyroid cells, potentially including the poorly understood inverse control of PTH secretion by Ca$^{2+}$-calmodulin, take place in caveolin-enriched plasma membrane domains.

**EXPERIMENTAL PROCEDURES**

**Materials**

Anti-PKCα antibody, Triton X-100, and protease inhibitors were from Boehringer Mannheim. The Renaissance kit was purchased from Du Pont. Anti-PKCα, γ, and δ antibodies and a kit containing 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) were from Life Technologies, Inc. Monoclonal and polyclonal anti-caveolin, anti-eNOS, and anti-PKCα and -δ antibodies were from Transduction Laboratories (Lexington, KY). Anti-Gαq/11 antibody was kindly provided by Dr. Armen H. Tashjian, Jr. (Harvard School of Public Health, Boston, MA). CaR-specific antisera were raised in rabbits against peptides based on the CaR sequence (antisera 4637 (FF) to amino acids 345–359 and antisera 4641 to amino acids 215–237) of the bovine parathyroid CaR. Both were generous gifts of NPS Pharmaceuticals, Inc. (Salt Lake City, UT). Ocyt chloride and peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Sigma.

**Methods**

**Cell Preparation and Incubations**—Dispersed bovine parathyroid cells were prepared by collagenase and DNase digestion as previously described (42). Cells were used immediately as acutely dispersed cells or were cultured overnight in Dulbecco's modified Eagle's medium (--F12 medium with penicillin and streptomycin. Cell culture medium was then aspirated, and the cells were washed with Dulbecco's modified Eagle's medium containing 0.5 mM Ca$^{2+}$, 0.5 mM Mg$^{2+}$, and 0.2% bovine serum albumin ("standard medium") and subsequently incubated with varying levels of Ca$^{2+}$ in standard medium as described below.

**Immunohistochemistry**—Frozen sections of bovine parathyroid gland (5-μm thickness) were air dried and fixed for 5 min with freshly prepared 2% formaldehyde in PBS. After treatment with an endogenous peroxidase inhibitor (DAKO Corp., Carpenteria, CA), the slides were blocked with 1% (w/v) bovine serum albumin in PBS for 30 min. Immunostaining was performed by sequential application of primary antibody (e.g. mouse anti-caveolin-1) and secondary antibody (peroxidase-conjugated goat anti-mouse IgG) in blocking solution and then the DAKO ABC substrate system as described before (11, 13). Alkaline phosphatase activity was demonstrated by direct staining of the slides with the NBT/BCIP kit according to the manufacturer's instructions (Life Technologies, Inc.). Photomicrographs were taken with 10× and 100× objectives.

**Preparation of Caveolin-enriched Membrane Fractions (CEMF)**—Bovine parathyroid gland CEMF were isolated using two OptiPrep density gradients as described by Smirnova et al. (26). The OptiPrep (7–18% protein) (26) were homogenized in 0.25 M sucrose, 1 mM EDTA, 20 mM HEPES, pH 7.4, with 10 μg/ml each of aprotinin, leupeptin, soybean trypsin inhibitor, and capsin enzyme inhibitor as well as 100 μg/ml of Pefabloc (11). First, phosphatase-sensitive plasma membranes (PM) by Percoll gradient fractionation. After removal of nuclei and cell debris by low speed centrifugation, the postnuclear supernatant (PNS) was layered onto 30% Percoll and sedimented at 84,000 × g for 30 min. The alkaline phosphatase-positive plasma membrane fraction was then disrupted by sonication. Caveolar membrane was separated from the remainder of the plasma membrane using two OptiPrep density gradients. The sonicated plasma membrane fractions were suspended in 23% OptiPrep and applied below a 20–10% OptiPrep gradient (OptiPrep 1) and sedimented at 18,000 × g for 90 min. (26). After determination of alkaline phosphatase (AP) activity and protein content, each sample from the first OptiPrep gradient was concentrated by trichloroacetic acid precipitation and analyzed by Western blot analysis as described below, or the top six AP-positive fractions were pooled and applied to a discontinuous flotation gradient (OptiPrep 2) to concentrate the caveolar membranes (26).

**Electrophoresis and Immunoblots**—Western blot analysis was performed as before (11). Briefly, samples were separated on 7.5% SDS-polyacrylamide gels (SDS-PAGE) according to Laemmli (43) or on linear 5–12% SDG gradient gels. The separated proteins were then transferred to nitrocellulose filters (Schleicher and Schuell), incubated with blocking solution (PBS with 0.25% Triton X-100 and 5% dry milk or 3% bovine serum albumin) for 1 h at room temperature, incubated for 1–3 h with primary and subsequently secondary antibodies diluted in blocking solution, and finally developed with the Renaissance ECL system as described by the manufacturer. Protein concentrations were measured using the Micro BCA protein reagent kit (Pierce) or the Bio-Rad DC assay (11, 26).

**Immunoprecipitation**—Parathyroid cells were washed with ice-cold PBS and lysed with immunoprecipitation buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, protease inhibitors (as described above), and the following detersgents: 60 mM octyl glucoside, 1% Triton X-100 (28) or 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS (32). The cell lysate was centrifuged at 10,000 × g for 10 min and preclotted with protein A-Sepharose CL-4B beads (Pharmacia) for 30 min at 4 °C. For immunoprecipitation, equal amounts of protein (150 μg of total cell lysate) were incubated with 5–10 μg of monoclonal or rabbit polyclonal antibodies for 1 h, followed by addition of anti-mouse IgG for 30 min (if monoclonal antibody was used). Protein A-Sepharose beads were then added for a further 1 h at 4 °C. Bound immune complexes were washed three times with immunoprecipitation buffer containing protease and phosphatase inhibitors and detersgents. The pelleted was eluted by boiling for 5 min with 2 × Laemmli sample buffer (14). Supernatant proteins were separated by SDS-PAGE as above, transferred to nitrocellulose filters, and immunoblotted with the indicated antibody.

**Alkaline Phosphatase Enzymatic Assay**—2 μl of each fraction from the OptiPrep gradient were spotted on nitrocellulose filter and developed using the AP substrate kit (NBT/BCIP). After development, the reaction was stopped by washing the filter with distilled water.

**RESULTS**

Alkaline Phosphatase and Caveolin Are Located on the Parathyroid Cell Surface—Frozen sections of bovine parathyroid glands stained for alkaline phosphatase activity (Fig. 1, A and C) show abundant punctate deposition of the insoluble reaction product solely on the surface of parathyroid chief cells, with little or no AP activity on endothelial cells (Fig. 1A). To determine if caveolin is present in parathyroid cells, we initially performed immunohistochemical analyses of bovine parathyroid...
Caveolin can associate with the endothelin type A receptor (28), binds inactive forms of some signaling proteins (i.e. Ras and G proteins), and provides a locus for sphingomyelin hydrolysis. The CaR is a G protein-coupled receptor (GPCR). Recently, two GPCRs, the muscarinic m2 and bradykinin B2 receptors, have been shown to be targeted to caveolae upon agonist stimulation (29, 30). These two receptors initiate signaling cascades resulting in NO production in several cell types, including endothelial cells and myocytes. We designed further studies, therefore, to determine whether eNOS is localized in CEMF of parathyroid cells. Subcellular fractionation of caveolae in parathyroid lysates showed that eNOS comigrates with caveolin-1 and the CaR (Fig. 2, A and B). Next, we performed co-immunoprecipitation and immunoblotting experiments to investigate interactions between caveolin, CaR, and eNOS. As shown in Fig. 3, A and B, the CaR and eNOS were specifically co-immunoprecipitated by anti-caveolin-1 antibody. To determine whether the interactions between caveolin-1 and the CaR as well as with eNOS are stable, cell lysates were solubilized with different detergents (Fig. 3, C and D) and subjected to immunoprecipitation with anti-CaR or anti-eNOS antibodies. The specifically bound proteins were detected by immunoblotting. Under both detergent conditions, CaR and eNOS co-immunoprecipitate with caveolin. The associations of the CaR and of eNOS with caveolin are specific as no immunoreactivity was detected when the anti-CaR antibody was preincubated with specific peptide (Fig. 3C) and non-immune mouse IgG was used as a control for the anti-caveolin antibody (Fig. 3, A and B).

**Other Signaling Molecules Found in Caveolae**—Because caveolae participate in GPCR signaling (18–20), we examined whether the CaR copurifies with Gaq/11 in parathyroid CEMF. Caveolae-like membranes were separated from the remainder of the plasma membrane using two OptiPrep density gradients as before (Fig. 2). Immunoblotting showed that CEMF from parathyroid glands. Fig. 1, B and D show the localization of caveolin-1 immunoreactivity on the plasma membrane of parathyroid chief cells and endothelial cells. Punctate staining for caveolin-1 was clearly observed on the cell surface at higher magnification. There was no staining after replacement of the first antibody with an equivalent dilution of mouse IgG (data not shown).

**CaR Associates with CEMF**—To determine whether the CaR is present in CEMF, bovine parathyroid glands were homogenized and fractionated using the non-detergent-based protocol of Smart et al. (26), which separates caveolin-rich membrane domains from the bulk of cellular membranes and cytosolic proteins. After isolation of PM by Percoll gradient fractionation from a PNS, CEMF are separated from the remainder of the plasma membrane using two OptiPrep density gradients (see "Experimental Procedures"). The first gradient (OptiPrep 1) separates the bulk of the plasma membrane proteins from CEMF. Protein samples (10 μg) from OptiPrep 1 fractions separated by electrophoresis and immunoblotted with anti-CaR antisera and with anti-caveolin-1 IgG revealed that CaR protein was highly enriched in the caveolar fraction, but only 23% of the PM protein was in CEMF (Fig. 2, A and C). We then pooled the top 6 AP-containing fractions and used a discontinuous gradient (OptiPrep 2) to obtain a highly enriched fraction of caveolin-containing membranes. Distributions of the CaR and caveolin proteins were detected by immunoblot. Equal amounts (5 μg) of protein from PNS, PM fractions isolated by Percoll gradient fractionation, and CEMF (Cav) isolated by OptiPrep 2 were separated by electrophoresis and immunoblotted with anti-CaR (4641) antisera and anti-caveolin-1 IgG. The immunostaining was specific because it was ablated following preabsorption of the antibody with specific peptide (not shown). In CEMF as well as in PM and PNS, Anti-CaR antisera showed a CaR-immunoreactive doublet at about 130–160 kDa (corresponding to the CaR monomer) and higher molecular weight forms. Based on the apparent molecular weights of 300 and 330 (as determined on 4% SDS-PAGE, data not shown), the higher molecular weight forms could represent CaR dimer. Formation of these dimeric forms requires a thiol-sensitive bond (not shown). CaR protein was highly enriched in the caveolar membrane domains (Fig. 2B, Cav) with approximately 6.7 ± 2.1- and 22.1 ± 0.8-fold enrichments in CEMF relative to plasma membrane and postnuclear supernatant, respectively, for the putative dimeric form of the receptor (~300–330 kDa) (mean ± SEM for three determinations). There were 6.7 ± 2.4- and 6.3 ± 0.9-fold enrichments of CaR protein in CEMF relative to PM and PNS, respectively, for the monomeric form of the CaR (at ~130–160 kDa) (mean ± SEM for three determinations).

**CaR and eNOS Reside in CEMF**—Caveolin can associate with the endothelin type A receptor (28), binds inactive forms of some signaling proteins (i.e. Ras and G proteins), and provides a locus for sphingomyelin hydrolysis. The CaR is a G protein-coupled receptor (GPCR). Recently, two GPCRs, the muscarinic m2 and bradykinin B2 receptors, have been shown to be targeted to caveolae upon agonist stimulation (29, 30). These two receptors initiate signaling cascades resulting in NO production in several cell types, including endothelial cells and myocytes. We designed further studies, therefore, to determine whether eNOS is localized in CEMF of parathyroid cells. Subcellular fractionation of caveolae in parathyroid lysates showed that eNOS comigrates with caveolin-1 and the CaR (Fig. 2, A and B). Next, we performed co-immunoprecipitation and immunoblotting experiments to investigate interactions between caveolin, CaR, and eNOS. As shown in Fig. 3, A and B, the CaR and eNOS were specifically co-immunoprecipitated by anti-caveolin-1 antibody. To determine whether the interactions between caveolin-1 and the CaR as well as with eNOS are stable, cell lysates were solubilized with different detergents (Fig. 3, C and D) and subjected to immunoprecipitation with anti-CaR or anti-eNOS antibodies. The specifically bound proteins were detected by immunoblotting. Under both detergent conditions, CaR and eNOS co-immunoprecipitate with caveolin. The associations of the CaR and of eNOS with caveolin are specific as no immunoreactivity was detected when the anti-CaR antibody was preincubated with specific peptide (Fig. 3C) and non-immune mouse IgG was used as a control for the anti-caveolin antibody (Fig. 3, A and B).
OptiPrep 2 are highly enriched in caveolin and Goq11 compared with the postnuclear supernatant (Fig. 4). Other key signaling molecules are also present in these caveolin-rich membrane domains, including PKC isoforms. Samples from whole cell lysates (25 μg/lane), the postnuclear fraction (5 μg/lane), and CEMF (5 μg/lane) were subjected to SDS-PAGE, transferred to membranes, and then probed with the antibody of interest. Fig. 4 shows that the caveolin-like fractions are enriched in PKCa, -δ, and -ζ but not in PKCγ. Co-immunoprecipitation revealed that the CaR and caveolin as well as Goq11, PKCa, and PKCζ form a stable complex in a mixture of Triton X-100 and octylglucoside (Fig. 5).

High Ca\(^{2+}\) Increases Tyrosine Phosphorylation of Caveolin in Caveolar Fractions—High Ca\(^{2+}\) induces changes in several second messengers and suppresses PTH secretion in parathyroid cells (1–3, 14). Therefore, we tested whether Ca\(^{2+}\) also modulates caveolin in parathyroid cell CEMF. Caveolin was originally isolated as a phosphotyrosine-containing protein in Src-transformed chick embryo fibroblasts, and phosphorylated caveolin has altered properties (20, 39, 40, 44). Therefore, we immunoprecipitated caveolin following incubation of parathyroid cells with varying levels of Ca\(^{2+}\) to determine the effect of Ca\(^{2+}\) on the tyrosine phosphorylation of caveolin. Fig. 6 shows that the intensity of the band comigrating with caveolin that is immunostained with anti-phosphotyrosine antibody was 5.2-
fold greater in cells incubated with 3.0 mM than with 0.5 mM Ca\[^{2+}\]_o and when corrected for the amount of caveolin, it was 3.2-fold higher.

**DISCUSSION**

Parathyroid cells are secretory cells with the capacity to sense and be regulated by changes in Ca\[^{2+}\]_o through a receptor-mediated mechanism. We have recently cloned and characterized an extracellular calcium-sensing receptor, CaR, from bovine parathyroid that plays a central role in the sensing of Ca\[^{2+}\]_o by parathyroid, kidney, and other cell types (1, 4–7). This receptor has a large extracellular amino-terminal domain, seven predicted membrane-spanning segments, and a smaller intracellular domain (4), identifying it as a member of the superfamily of GPCRs.

The secretory apparatus of parathyroid cells is inversely regulated by acute elevations of Ca\[^{2+}\]_o, a process mediated by the CaR. Inhibition of PTH secretion occurs within seconds following a rise in Ca\[^{2+}\]_o despite a concomitant rise in Ca\[^{2+}\]_i (1–3), a stimulus for exocytosis in the vast majority of secretory cells. Thus, either the secretory pathway of parathyroid cells is designed to respond to rises in Ca\[^{2+}\]_o, in an inverse fashion (i.e. with a block of secretory vesicle release) or additional, receptor-mediated events ultimately inhibit late secretory steps. The same CaR stimulates secretion by similar activation of second messengers in other CaR-expressing cells (i.e. C-cells) (7), suggesting that as yet poorly defined distal effectors are responsible for the inverse regulation of secretion by the CaR in parathyroid cells. It is possible that the site of CaR activation plays a role in the inverse regulation of PTH release.

Parathyroid chief cells appear polarized in intact parathyroid glands, with an apicolateral localization of ATPases, alkaline phosphatase, and 5'-nucleotidase (16, 17). In the present study, we observed staining in the cell membranes of parathyroid chief cells using immunohistochemistry with anti-caveolin antibodies in frozen sections of bovine parathyroid glands. Caveolin immunoreactivity exhibited a punctate, cell surface distribution in bovine parathyroid chief cells. Parathyroid glands have a rich capillary network (45), and endothelial cells are rich in caveolae coated with caveolin. Immunostaining of parathyroid glands with an anti-caveolin antibody showed staining of both endothelial cells and parathyroid cells. Parathyroid cells also stained strongly for AP, in contrast to endothelial cells, which showed minimal staining.

Controversy has surrounded the localization of certain proteins in caveolae (46). CEMF from bovine parathyroid glands were isolated by the detergent-free method of Smart, et al. (26) and by immunoprecipitation with anti-caveolin antibodies (26, 32). Our results show that parathyroid cell CEMF are enriched in caveolin, the CaR, and alkaline phosphatase. Some GPCRs, such as the muscarinic acetylcholine and β₂-adrenergic receptors, are uniformly distributed throughout the plasma membrane and may translocate to caveolae upon agonist binding (28, 30, 48). The G protein-coupled endothelin receptor subtype A and its bound endothelin ligand are found together in caveolae (28). In contrast, exposure of Rat-1 cells to epidermal growth factor induces translocation of this tyrosine kinase receptor away from caveolae (32). Here, we show that at least a substantial fraction of the total CaR content resides within parathyroid CEMF independent of the level of its principal agonist, Ca\[^{2+}\]_o as assessed by immunohistochemical and biochemical approaches. In addition, co-immunoprecipitation showed that the CaR in parathyroid cells is associated with caveolin. Recently, G protein subunits have been shown to interact with caveolin (26, 34, 44) and are highly concentrated in caveolar membranes purified from diverse sources (18, 26, 34, 35). When activated by high Ca\[^{2+}\]_o, the CaR stimulates phospholipase C via a pertussis toxin-insensitive G protein and mobilizes Ca\[^{2+}\]. Studies in parathyroid cells have demonstrated the presence of several G protein α subunits, including those for G\(_{11}\) and G\(_{q/11}\) (14). Here, we show that the CaR in parathyroid caveolin-rich plasma membrane colocalizes with G\(_{q/11}\).

NOS is a cell type-specific enzyme catalyzing nitric oxide synthesis. NO is a short-lived radical that transmits cellular signals involved in vasorelaxation, neurotransmission, and cytotoxicity (36, 37, 47). eNOS is a constitutive enzyme localized in plasmalemmal caveolae of endothelial cells that is activated by agonists increasing Ca\[^{2+}\]_o. Caveolin-1 interacts directly with eNOS and inhibits its catalytic activity (37, 38, 47). Our biochemical approaches show that parathyroid cell CEMF are enriched in eNOS. In addition, eNOS co-immunoprecipitates with caveolin-1 and the CaR. Sodium nitroprusside, an NO donor, significantly reduced intracellular cAMP accumulation and parathyroid hormone release in dispersed bovine parathyroid cells (48). Sodium nitroprusside is a potent vasodilator stimulating guanylate cyclase activity and cGMP accumulation (49). NO exerts many of its biological actions by activating soluble guanylyl cyclase and stimulating cGMP production (49). Thus, activation of eNOS and NO production may participate in transmembrane signaling in parathyroid cells and could contribute to suppression of PTH secretion at high Ca\[^{2+}\].

Several types of receptors are activated through ligand-induced dimerization or oligomerization (50–52). Dimerization provides both specificity and flexibility in ligand binding be-
cause of the opportunity for the assembly of different homo- and heterodimeric receptors, depending on which receptors and signal transducers are expressed by a particular cell type (50–52). Exposure to ligand induces receptor dimerization, leading to their autophosphorylation, a step necessary for activation of intracellular signaling (52). The CaR in bovine parathyroid CEMF seems to be in the form of a dimer. The mechanism(s) underlying formation of CaR dimers remains to be fully characterized; moreover, it is not currently known whether dimerization activates or inactivates or does not affect CaR activity.

Calvin-sensing Receptor, Caveolae, and Parathyroid Cells

4. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, D., Butters, R., Kifor, O., Sun, A., Hedger, M. A., Lytton, J., and Hebert, S. C. (1993) Nature 366, 575–580
5. Chattopadhyay, N., Mithal, A., and Brown, E. M. (1996) Endocr. Rev. 17, 289–307
6. Riccardi, D., Park, J., Lee, W., Gamba, G., Brown, E. M., and Hebert, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 131–135
7. Garrett, J. E., Tamir, H., Kifor, O., Simin, R. T., Rogers, K. V., Mithal, A., Gagel, R. F., and Brown, E. M. (1995) Endocrinology 136, 5202–5211
8. Bai, M., Gamba, G., Riccardi, D., Kifor, O., Sun, A., Hebert, S., and Brown, E. M. (1996) J. Biol. Chem. 271, 15357–15354
9. Pearce, S., Trump, D., Wooding, C., Besser, G., Chew, S., Heath, D., Hughes, I., and Thakker, R. (1995) J. Clin. Invest. 95, 736–740
10. Gaglin, P., Giachmann, F., Bazzaro, M., Giovannini, M., Goureau, Y., Sarfati, E., and Drueke, T. (1996) Kidney Int. 51, 328–336
11. Kifor, O., Moore, F. D., Wang, P., Goldstein, M., Vassilev, P., Kifor, I., Hebert, S. C., and Brown, E. M. (1996) J. Clin. Endocrinol. Metab. 81, 1598–1606
12. Fox, J., Petty, B. A., and Nemeth, E. F. (1997) J. Bone Miner. Res. 12, 715–725
13. Varrault, A., Rodriguez-Penasa, M. S., Goldsmith, P. K., Mithal, A., Brown, E. M., and Spiegel, A. M. (1995) Endocrinology 136, 4390–4398
14. Wild, P., and Schrader, E. M. (1996) Histochemistry 94, 409–414
15. Tsuchiya, T., and Tamate, H. (1980) Histochemistry 65, 321–323
16. Parton, R. G., Joggerst, B., and Simons, K. (1994) J. Biol. Chem. 269, 1199–1215
17. Ying, Y. S., Anderson, R. G. W., and Drueke, T. G. (1996) Cold Spring Harbor Symp. Quant. Biol. 57, 593–604
18. Lisanti, M. P., Scherer, P. E., Tang, Z. L., and Sargsiago, M. (1994) Trends Cell Biol. 4, 231–235
19. Anderson, R. G. W. (1995) Curr. Opin. Cell Biol. 5, 647–652
20. Huang, C., Hepler, J. R., Chen, L. T., Gilman, A. G., Anderson, R. G. W., and Mumbey, S. M. (1997) Mol. Cell Biol. 7, 2365–2378
21. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
22. Smart, E. J., Ying, Y., Donzell, W. C., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 29427–29435
23. Liu, P., and Anderson, R. G. W. (1995) J. Biol. Chem. 270, 27179–27185
24. Smart, E. J., Ying, Y. S., Anderson, R. G. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 92, 10104–10108
25. Liu, P., Ying, Y., Ko, Y. G., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 10299–10303
26. Chun, M., Lianyuan, U. K., Lisanti, M. P., and Lodish, H. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11728–11732
27. de Weerd, W. F. C., and Leeb-Lundberg, L. M. F. (1997) J. Biol. Chem. 272, 17855–17866
28. Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997) J. Biol. Chem. 272, 17744–17748
29. Smart, E. J., Ying, Y. S., and Anderson, R. G. W. (1995) J. Biol. Chem. 270, 14399–14404
30. Mineo, C, Jaken, S., and Anderson, R. G. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9407–9411
31. Bevers, E. M., Pollak, M., Seidman, C. E., Seidman, C. E., Chu, Y. W., Riccardi, D., and Hebert, S. C. (1995) Nature 372, 340–340
32. Gardner, D. G., Brown, E. M., and Aurbach, G. D. (1979) J. Clin. Invest. 64, 970–978
33. Smart, E. J., Ying, Y., Donzell, W. C., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 11930–11935
34. Sargiacomo, M., Scherer, P. E., Tang, Z. L., and Sargsiago, M. (1994) Trends Cell Biol. 4, 239–245
35. Song, K. S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1995) J. Biol. Chem. 270, 2477–2484
36. Tanaka, T., Otsuka, A. J., and Boontrakulpoontawee, P., Katada, T., Otsuka A. J., and Fujimoto, T., and Fujita, T., and Kumaia, A. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5009–5014
37. Brown, E. M., Hurwitz, S., and Aurbach, G. D. (1976) Endocrinology 99, 1582–1585
38. Laemmli, U. K. (1970) Nature 227, 680–685
39. Liu, D. H., Horner, T., Rogers, R. A., and Schnitzer, J. E. (1997) J. Biol. Chem. 272, 7121–7122
40. Wild, P., and Setoguti, T. (1995) Microsc. Res. Tech. 32, 120–128
41. Parton, R. G., and Simons, K. (1994) J. Biol. Chem. 269, 1398–1399
42. Michel, T., and Feron, O. (1997) J. Clin. Invest. 99, 2146–2152
43. Gardner, D. G., Brown, E. M., and Aurbach, G. D. (1979) Endocrinology 105, 360–366
44. Brown, E. M., and Iadecola, C. (1996) Methods Enzymol. 260, 468–462
45. Heldin, C.-H. (1995) Cell 80, 213–223
46. Leof, B. S., Coteehica, S., Samama, P., and Costa, T. (1993) Trends Pharmacol. Sci. 14, 303–309
47. Cevey, S., and Devi L. A. (1997) J. Biol. Chem. 272, 29599–29604
48. McNeil, S. E., Hobson, S. A., Nipper, V., and Rodland, K. D. (1998) J. Biol. Chem. 273, 1114–1120
49. Mineo, C, Jaken, S., and Anderson, R. G. W. (1996) Mol. Biol. Cell 7, (suppl.) 1, S181 (abstr.)
50. Oka, N., Yamamoto, M., Schwencke, C., Kawabe, J., Ebina, T., Ohno, S., Couet, J., Lisanti, M. P., and Ishikawa, Y. (1997) J. Biol. Chem. 272, 33416–33421

O. Kifor and E. M. Brown, unpublished observations.