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Role of the cytoskeleton in extracellular calcium-regulated PTH release

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Abstract

The calcium-sensing receptor (CaR) mediates the effects of extracellular calcium ($[Ca^{2+}]_o$) on PTH release, such that increasing levels of $[Ca^{2+}]_o$ inhibit PTH secretion through poorly defined mechanisms. In the present studies, immunocytochemical analysis demonstrated that F-actin, PTH, CaR, and caveolin-1 are colocalized at the apical secretory pole of PT cells, and subcellular fractionation of PT cells showed these proteins to be present within the secretory granule fraction. High $[Ca^{2+}]_o$ caused F-actin, PTH, and caveolin-1 to move to the apical pole of the cells. Depolymerization of F-actin by cytochalasin reduced the actin network and induced redistribution of actin/caveolin-1 to a dispersed pattern within the cell. The F-actin-severing compounds, latrunculin and cytochalasin, significantly increased PTH secretion, while the actin polymerizing agent, jasplakinolide, substantially inhibited PTH secretion. We have demonstrated that in polarized PT cells, the F-actin cytoskeleton is involved in the regulation of PTH secretion and is critical for inhibition of PTH secretion by high calcium.

Keywords: Calcium-sensing receptor; PTH; Calcium; Cytoskeleton; Parathyroid cells

The parathyroid (PT) glands play a central role in calcium homeostasis by modulating parathyroid hormone (PTH) secretion in response to small changes in the extracellular free Ca^{2+}concentration ($[Ca^{2+}]_o$) [1]. Interestingly, CaR agonists activate transduction pathways that typically increase hormone secretion but result in inhibition of PTH secretion in PT cells [2]. Regulated exocytosis is the process by which a physiologic stimulus leads to the fusion of storage vesicles with the plasma membrane and the subsequent release of secretory products into the extracellular space [8]. In most secretory cells, the cytoplasmic face of the apical membrane is closely associated with a dense network of actin filaments and actin-binding proteins [9,10]. During cell activation, this dense F-actin network is depolymerized to allow fusion of secretory vesicles with the plasma membrane, often associated with a rise in the cytosolic calcium concentration $[Ca^{2+}]_i$. Caveolae are flask-like invaginations of the plasma membrane that serve as repositories for signaling molecules, tend to collect in actin-rich regions of secretory cells, and contain a number of actin-binding proteins, including filamin-A, which can interact directly with both caveolin-1 and actin [11,12]. We previously showed that PT cells express caveolin-1, that caveolae are the major site of localization of the cell surface CaR, and that filamin-A is a binding partner of the CaR [15–17]. In this paper we show that actin polymerization and depolymerization appear to play a critical role in the regulation of PTH secretion. These findings demonstrate that actin, caveolin-1, filamin A, CaR, and PTH colocalize at the apical secretory pole of PT cells, where the secretory vesicles interact with the actin cytoskeleton to play a key role in $[Ca^{2+}]_o$-regulated PTH release.

Materials and methods

Materials. Protease-free bovine serum albumin (BSA) and protease inhibitors were from Roche (Indianapolis, IN). We obtained the Renaissance ECL system from Perkin-Elmer Life Science Products

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(Boston, MA). Cell culture medium (DMEM Ham's F-12), 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), and nitroblue tetrazolium chloride (NBT) were purchased from Gibco-BRL (Gaithersburg, MD). Monoclonal antibodies included those to caveolin-1, caveolin-2, and phospho-caveolin from BD Biosciences Transduction Laboratories (Lexington, KY). A polyclonal antibody to caveolin-1 was from Santa Cruz Biochemicals (San Francisco, CA). A rat PTH antibody (directed to the N-terminus) and a monoclonal antibody to chromogranin A were from NeoMarkers (Fremont, CA), and a goat antibody to the PTH C-terminus was from CHEMICON (Temecula, CA). A monoclonal anti-filamin-A antibody was a gift of Drs. John Hartwig and Thomas Stossel (Brigham and Women's Hospital, Boston MA). Biotinylated anti-mouse IgG and Vectashield mounting medium were from Vector Lab. (Burlingame, CA). CaR-specific polyclonal antisera to peptides based on the CaR sequence were raised in rabbits. Antiserum 4637 produced to amino acids 345–359 was a generous gift of NPS Pharmaceuticals (Salt Lake City, UT). Goat anti-mouse IgG coupled to Alexa 568 and goat anti-rabbit IgG coupled to Alexa 488 were purchased from Molecular Probes (Eugene, OR). The TSA™ Cyanine 5 system was from Perkin-Elmer (Boston, MA).

Cell preparation and incubations. Dispersed bovine PT cells were prepared by collagenase and deoxyribonuclease digestion of glands as described previously [17]. For determinations of PTH release, PT cells were used immediately as acutely dispersed cells. The cells were washed with Eagle's minimal essential medium (EMEM) containing 0.5 mM $[Ca^{2+}]_o$, 0.5 mM $[Mg^{2+}]_o$, and 0.2% BSA, and were subsequently incubated at 37 °C with various concentrations of $[Ca^{2+}]_o$ or other additives as detailed in Results.

Immunocytochemistry. Dispersed PT cells were allowed to attach to glass coverslips coated with or without Matrigel (Becton–Dickinson, Bedford, MA), and the adherent cells were incubated at 37 °C. The cells were fixed, permeabilized, and incubated with blocking solution as described previously [16,17]. For triple immunofluorescence staining, the slides were incubated with a mixture of primary antibodies in blocking solution overnight at 4 °C in a humidified chamber. Fluorescence images were viewed using the 100× objective of a Bio-Rad MRC 1024/2P multiphoton microscope equipped with krypton and argon lasers at the Brigham and Women's Hospital Confocal Microscopy Core facility.

Purification of caveolin-rich membrane fractions. Fractions enriched in caveolin were purified as described previously [16,17]. Fractions (1.3 ml) were removed sequentially from the top of the gradient and designated as fractions 1–9 (fractions 2–3 and 6–9 are considered to be of Triton X-100-insoluble caveolar and Triton X-100-soluble non-caveolar origin, respectively). Fractions were subjected to SDS–PAGE and immunoblotting or to PTH determination.

Electrophoresis and immunoblots. Western blot analysis was performed essentially as described previously [15–17]. After preparation of fractions enriched in caveolin-1, equal amounts of protein were separated by SDS–PAGE and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH). The blots were subsequently incubated with blocking solution (PBS with 0.25% Triton X-100 and 5% non-fat dry milk) for 1 h at room temperature. The membranes were incubated overnight with primary antiserum and, after washing, with secondary antibodies. The bands were visualized by chemiluminescence. Protein concentration was measured with Micro BCA protein reagent kit (Pierce Chemical Co., Rockford, IL).

Determination of Ca^{2+}-regulated PTH release. Dispersed BPT cells were incubated with 0.5 mM $[Ca^{2+}]_o$ and varying concentration of $[Ca^{2+}]_o$ (0.5–2.0 or 3.0 mM) for 1 h at 37 °C. PTH released into the medium from BPT cells was quantified using a whole rat PTH immunoradiometric assay kit (ALPCO, Salem, NH).

Statistics. The data are presented as means ± SEM of the indicated number of experiments. Statistical software was used to analyze the results by one-way analysis of variance (ANOVA) with the Student–Newman–Keuls Method. A p value of <0.05 was considered to indicate a statistically significant difference.

Results

The regulated secretory pathway in PT cells

Our previous work showed localization of the CaR in the caveolin-1-rich fraction of the plasma membrane of bovine parathyroid cells (BPT) [15–17], suggesting a close association with the secretory pathway in PT cells. Here we analyzed the localization of the CaR relative to that of PTH in BPT cells incubated with low or high $[Ca^{2+}]_o$ (Fig. 1). At low $[Ca^{2+}]_o$, the immunostaining of the CaR is distributed both in the plasma membrane, particularly

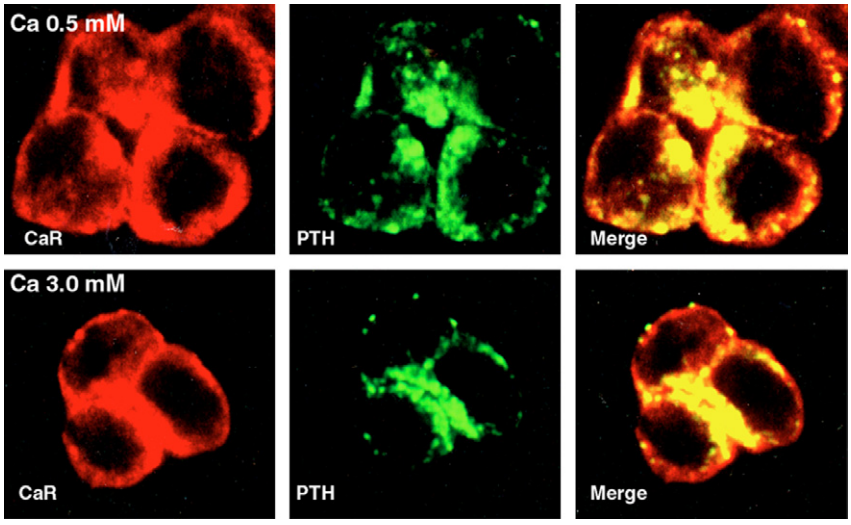

Fig. 1. Confocal analysis detects apical surface expression of CaR and PTH. PT cells were incubated with 0.5 or 3.0 mM $[Ca^{2+}]_o$ in standard medium for 10 min, fixed and stained for the CaR (red) and PTH (green). Yellow color in Merge indicates colocalization of CaR and PTH. Magnification is 1000×. The data are representative of 3 separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

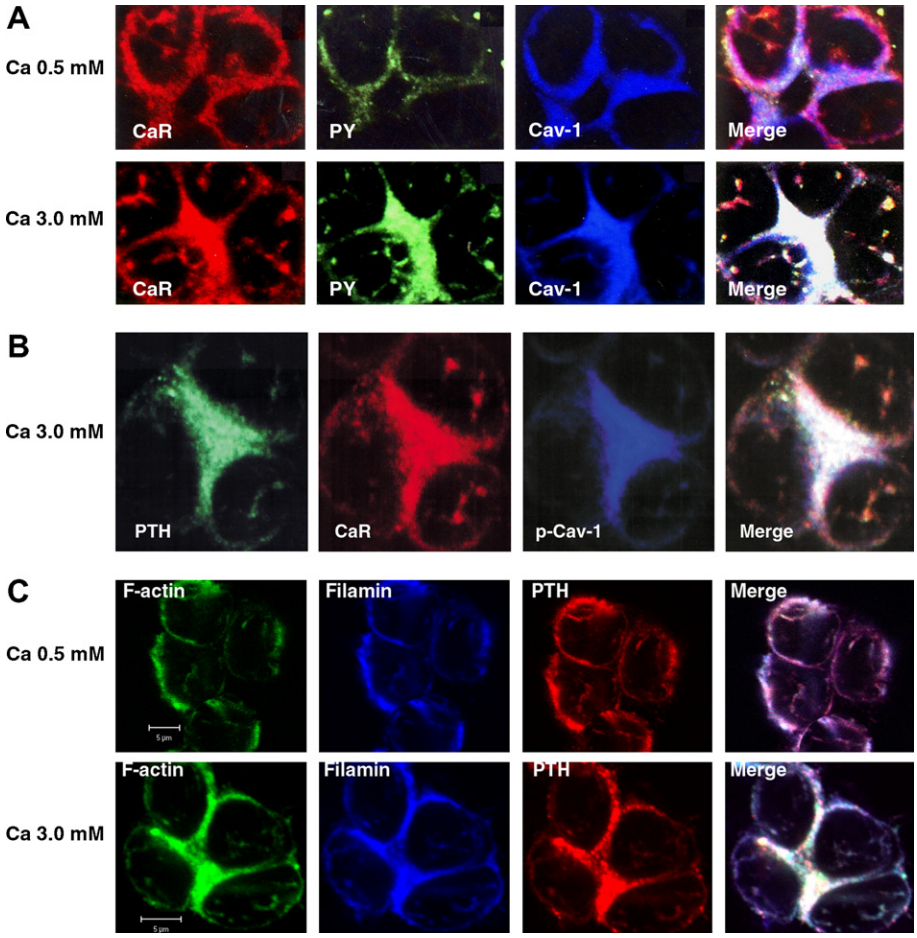

Fig. 2. High $[Ca^{2+}]_o$ increases the accumulation of tyrosine-phosphorylated proteins, filamin-A, F-actin, and PTH at the apical pole of PT cells. PT cells were incubated with 0.5 or 3.0 mM $[Ca^{2+}]_o$ for 10 min and immunostained for the CaR, phospho-tyrosine (PY), and caveolin-1 (cav-1). (A) High $[Ca^{2+}]_o$ increased PY accumulation at the apical pole of the cells, where it was colocalized with caveolin-1 and the CaR (white color in Merge). (B) Cells incubated with high $[Ca^{2+}]_o$ were immunostained for PTH, CaR, and phosphorylated caveolin (pCav). Phosphorylated caveolin co-localizes with PTH and the CaR at the secretory pole of the cells. (C) PT cells were incubated with 0.5 or 3 mM $[Ca^{2+}]_o$ for 10 min and immunostained for filamin-A, F-actin (fluorescein-phalloidin), and PTH. Co-localization (white color) was visualized by merging the three images. The data are representative of two or three separate experiments.

at the secretory pole of the cells, as well as intracellularly. A substantial fraction of PTH is found at the apical pole, where PTH and the CaR are colocalized at the plasma membrane and in secretory granules (yellow color). At high $[Ca^{2+}]_o$, most of the colocalization is at the apical pole of the cells, where the cells are in close apposition, occluding the intercellular spaces present at low $[Ca^{2+}]_o$ (Figs. 1 and 2). We also found co-localization of caveolin-1 and chromogranin A, a marker for the secretory pathway, with PTH at the apical secretory pole consistent with the presence of both CaR and caveolin-1 in PTH-containing secretory granules (data not shown).

Recovery of CgA, PTH, and actin in caveolin-1-containing, Triton X-100-insoluble microdomains

To determine the relationship of secretory granules to caveolin-1-containing microdomains, we examined the distribution of PTH and CgA isolated from PT glands solubilized in cold Triton X-100 to isolate caveolae/rafts [5,16]. While a large amount of intact intracellular PTH was associated with the Triton X-100-soluble fractions, a substantial amount of intact intracellular PTH also floated in the Triton X-100 insoluble, caveolin-1-rich fraction. In addition, we found that immunoreactivity of CgA and actin were higher in detergent-insoluble caveolar fractions prepared from PT cells incubated with 2 mM $[Ca^{2+}]_o$ than in those incubated with 0.5 mM $[Ca^{2+}]_o$ (data not shown). Furthermore, more actin was associated with PTH in both Triton-insoluble and Triton-soluble fractions at high $[Ca^{2+}]_o$, indicating the high calcium might increase the association of the granules with the actin-based cytoskeleton.

Effect of high $[Ca^{2+}]_o$ on tyrosine phosphorylation and localization of various proteins in PT Cells

Tyrosine phosphorylation of proteins can induce conformational changes used in transduction pathways to modify their activity, alter their interactions with other

cellular proteins and regulate entry into the secretory pathway [13,19]. For example, we previously observed that high $[Ca^{2+}]_o$ stimulated tyrosine phosphorylation of ERK1/2 and caveolin-1, which bind to each other [15,16]. Therefore, we determined the distribution of phospho-tyrosine (PY) in PT cells incubated with low or high $[Ca^{2+}]_o$. High $[Ca^{2+}]_o$ increased PY accumulation as well as co-localization of PTH and CaR with p-caveolin at the apical pole of the PT cells (Fig. 2A and B). General PY immunostaining is concentrated at the secretory pole of the cells, and also found along the cell periphery. In contrast, p-caveolin in Fig. 2B is present as a dense aggregate exclusively at the secretory poles of the cells—a pattern of localization similar to that of filamin-A and F-actin (see below).

We also analyzed the effects of high $[Ca^{2+}]_o$ on the cellular distribution of filamin-A and F-actin (Fig. 2C). We have shown that filamin-A directly binds the C-tail of the bovine CaR in a region that differs from that mediating filamin-A's binding to caveolin-1 [20], suggesting a molecular complex involving caveolin-1, the CaR and F-actin. We analyzed the effects of high $[Ca^{2+}]_o$ on the relationship between filamin-A, F-actin, and PTH and found that high $[Ca^{2+}]_o$ increased the content of F-actin and filamin-A at the apical secretory pole of the cells and induced colocalization of F-actin, filamin-A, and PTH (Fig. 2C). Thus, an increase in $[Ca^{2+}]_o$ induces the redistribution of several proteins potentially involved in $[Ca^{2+}]_o$-regulated PTH release to the secretory poles of PT cells.

Regulation of cortical actin-caveolin-1 assembly in PT cells

Plasma membrane caveolae represent a stable compartment that is anchored to the actin cytoskeleton, and treatment of cells with the actin-depolymerizing agent, cytochalasin D, leads to increased lateral mobility of caveolin and clustering of caveolae [9–11]. Dispersed PT cells incubated with 0.5 mM $[Ca^{2+}]_o$ (a condition leading to a high rate of PTH secretion) showed heterogeneous immunostaining for F-actin and caveolin-1 that was distributed along much of the cell periphery as well as at the secretory poles of the cells (Fig. 3). In cells treated with 3.0 mM $[Ca^{2+}]_o$ (a condition suppressing PTH release), caveolin-1 co-localized with F-actin at the apical pole of the cells, producing a large, dense actin web (Fig. 3). Cytochalasin B dramatically reduced and disrupted the F-actin web in cells treated with 3.0 mM $[Ca^{2+}]_o$ and dissociated the F-actin and caveolin-1 complex. Cytochalasin B had the same effects in cells incubated with 0.5 mM $[Ca^{2+}]_o$, although the change was less pronounced (data not shown).

Involvement of the actin cytoskeleton in PTH secretion

The actin depolymerizing agents, latrunculin B and cytochalasin D, and the actin polymerizing agent, jasplakinolide, were used in PT cells to further study the role of the cytoskeleton in PTH secretion. Incubation of PT cells with actin-depolymerizing agents enhanced PTH secretion at

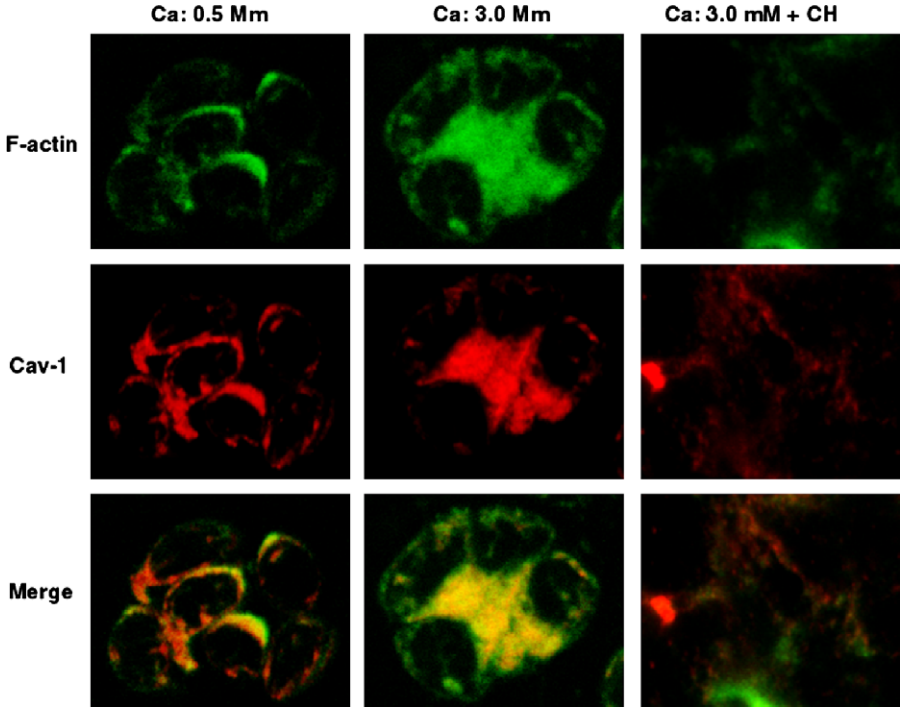

Fig. 3. Regulation of assembly of F-actin/caveolin-1 in PT cells. PT cells were treated with 5 μg of cytochalasin B for 1 h and incubated with 3 mM $[Ca^{2+}]_o$ for 30 min. For comparative experiments, cells were incubated for 30 min with 0.5 or 3 mM $[Ca^{2+}]_o$ without cytochalasin B. After permeabilization and fixation, the cells were immunostained with an antibody to caveolin-1 and fluorescein-phalloidin for F-actin. Yellow color in Merge represents colocalization of F-actin and caveolin-1. The data are representative of three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

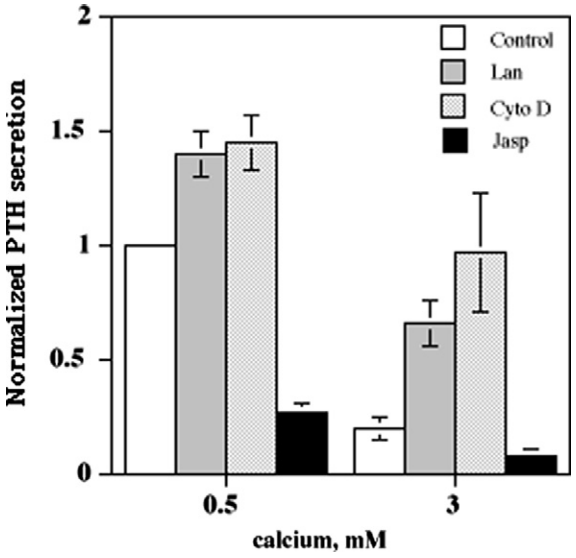

Fig. 4. Effect of actin depolymerization or polymerization on $[Ca^{2+}]_o$-regulated PTH release. Bovine PT cells were treated with or without 10 μM latrunculin A (Lan, actin depolymerizer), 30 μM cytochalasin D (Cyto D, actin depolymerizer) or 1 μM jasplakinolide (Jasp, actin polymerizer) for 60 min at 37 °C, then incubated for an additional 1 h with 0.5 or 3 mM $[Ca^{2+}]_o$. Supernatant samples were collected and assayed for PTH. The secretion of PTH was normalized to the values measured at 0.5 mM $[Ca^{2+}]_o$ under control conditions. Data values were the means ± SEM for 3–10 experiments. PTH secretion in the presence of latrunculin A or cytochalasin D was significantly greater than control values at 0.5 and 3.0 mM calcium ($p < 0.05$). PTH secretion in the presence of jasplakinolide was significantly reduced compared to control values at 0.5 and 3.0 mM calcium ($p < 0.05$).

both low and high $[Ca^{2+}]_o$, while jasplakinolide inhibited PTH secretion at both low and high $[Ca^{2+}]_o$ (Fig. 4). These data suggest that actin polymerization is important for the inhibition of PTH secretion at high $[Ca^{2+}]_o$.

Discussion

Caveolae are specialized lipid rafts organized by the cholesterol-binding protein, caveolin, which contain the CaR and various signaling molecules in PT cells [15–17]. In the presence of high $[Ca^{2+}]_o$, the CaR move to the caveolae and signal transduction increases therein [15–17]. While the function of caveolae in PTH secretion has not been elucidated, we observed variably reduced levels of caveolin-1 in PT cells prepared from human PT adenomas and showed that caveolin-1-negative cells exhibit reduced suppressibility of PTH secretion by high $[Ca^{2+}]_o$ [17]. These findings suggested that caveolin-1-containing microdomains might have important implications for CaR-mediated signaling pathways in PT cells and their coupling to inhibition of PTH secretion by high $[Ca^{2+}]_o$.

PT cells in situ are polar cells, with the Golgi complex and the secretory granules localized at the apical pole [18]. The secretory product is released by exocytosis at the apicolateral domain of the plasma membrane into the intercellular space; thus apical targeting of secretory granules is important for release of PTH. Caveolin-1 targeting to the secretory pathway is observed in exocrine secretory cells and some endocrine cells [6,7,21]. Similarly, we found an association of PTH and CgA with caveolin-1-containing microdomains in PT cells.

The secretory granule marker protein, CgA, binds to secretory granule membranes via cholesterol-rich regions in other cell types [4] and we observed that CgA and PTH co-localize in the same secretory granules in PT cells. Furthermore, it is known that CgA is responsible for stabilization of PTH within secretory granules, and CgA is co-secreted with PTH in a calcium-regulated manner [3,23]. We found in this study that PTH, CgA, actin, and caveolin-1 associate with the Triton X-100-insoluable fractions prepared from PT cells, and that high $[Ca^{2+}]_o$ promoted greater localization of these proteins within these caveolin-rich fractions. Dispersed PT cells treated acutely with high $[Ca^{2+}]_o$ show substantial inhibition of PTH release. High $[Ca^{2+}]_o$ increases the association of PTH with CgA in detergent-insoluble, caveolin-1-rich fractions, and enhances the association of PTH with caveolin-1/p-caveolin-1 adjacent to the plasma membrane (Fig. 2).

Caveolar rafts are the preferred platforms for membrane-linked actin polymerization [14,22]. Actin filaments form a cortical network that separates secretory granules into a small releasable granule pool and a larger reserve pool. Actin also plays a critical role in cell polarity and in polarized transport of vesicles along the secretory pathway [8,9]. The actin web can provide a physical barrier to exocytosis, and reducing the actin web is known to facilitate hormone secretion [8,9]. Interestingly, caveolae are anchored to the cytoskeleton through filamin-A binding to actin and caveolin-1, suggesting that filamin-A is important for the immobility of plasma membrane caveolae [10,11]. In addition, filamin-A also binds to the carboxyl-terminal tail of the CaR, an interaction that contributes to CaR-mediated activation of transduction pathways in CaR-expressing cells [20]. The interaction of the CaR with filamin-A may also link the receptor to the regulation of the actin-based cytoskeleton, since the actin polymerizing action of filamin-A can be controlled by phosphorylation [10,11,24]. High $[Ca^{2+}]_o$ enhanced F-actin association with caveolin-1 in PT cells in the present studies and this actin-bound filamin-A could stabilize polymerized actin, tethering CaR-containing secretory granules to the cytoskeleton, thereby immobilizing apical secretory granules and forming a physical barrier to the further movement of secretory granules toward the plasma membrane. We observed a strong accumulation of phospho-tyrosine at the apical secretory pole of PT cells incubated with high $[Ca^{2+}]_o$ (Fig. 2), which could participate in recruiting actin, while the associated increase in filamin-A would lead to stabilization of F-actin.

F-actin can have both a positive and a negative role in regulated exocytosis [25]. Although exocytosis requires actin depolymerization, it cannot occur without a minimal actin structure [25]. Accordingly, our results showed that, in the presence of low $[Ca^{2+}]_o$, there was minimal, disas-

sembled F-actin within some cells, and in the presence of 3 mM $[Ca^{2+}]_o$, a dense F-actin web associated with caveolin-1 at the apical secretory pole (Figs. 2 and 3). Since PT cells secrete mostly from their apical surface, this thick F-actin web could, as noted above, inhibit exocytosis. Indeed, incubation with cytochalasin B produced dissociation of F-actin and caveolin-1 in cells incubated with high $[Ca^{2+}]_o$ (Fig. 3). While earlier studies reported variable effects of actin-depolymerizing agents on PTH secretion [26], we demonstrated here that actin-depolymerizing agents enhanced PTH secretion in the presence of both low and high $[Ca^{2+}]_o$, while an actin-polymerizing agent inhibited PTH secretion at both at low and high $[Ca^{2+}]_o$ (Fig. 4). These data strongly suggest that actin polymerization and de-polymerization are critical for the control of PTH secretion by $[Ca^{2+}]_o$.

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