Acidification of Serotonin-containing Secretory Vesicles Induced by a Plasma Membrane Calcium Receptor*

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Parafollicular (PF) cells secrete 5-hydroxytryptamine in response to increased extracellular Ca2† (↑[Ca2+]e). This stimulus causes Cl− channels in PF secretory vesicles to open, leading to vesicle acidification. PF cells express a plasmalemmal heptahelical receptor (CaR) that binds Ca2†, Gd3+, and Ba2+. We now report that the CaR mediates vesicle acidification. Ca2†, Gd3+, and Ba2+ induced vesicle acidification, which was independent of channel-mediated Ca2† entry. Agonist-induced vesicle acidification was blocked by pertussis toxin, inhibitors of phosphatidylinositol-phospholipase C, calmodulin, NO synthase, guanylyl cyclase, or protein kinase G. PF cells contained NO synthase immunoreactivity, and vesicles were acidified by NO donors and dibutyryl cGMP. [Ca2+]e and Gd3+ mobilized thapsigargin-sensitive intracellular Ca2+ stores. [35S]Gαi and [35S]Gαs were immunoprecipitated from PF membranes incubated with agonists in the presence of [35S]adenosine 5′-O-(thiotriphosphate). Labeling of Gαi but not Gαs was antagonized by pertussis toxin. Vesicles acidified in response to activation of protein kinase C; however, protein kinase C inhibition blocked calcium channel- but not CaR-dependent acidification. We propose the following signal transduction pathway: CaR → Gαi → phosphatidylinositol-phospholipase C → inositol 1,4,5-trisphosphate → ↑[Ca2+]i → Ca2+/calmodulin → NO synthase → NO → guanylyl cyclase → cGMP → protein kinase G → opens vesicular Cl− channel.

Two types of secretory vesicle are represented in synaptic terminals. One is the small (∼50 nm) synaptic vesicle (SV) that stores small molecule neurotransmitters (1). SVs can be replenished locally by endocytic recycling from the plasma membrane (2–4) and thus are derived from endosomes (5, 6). The other type of vesicle, which is also found in neuroendocrine cells, is a large, dense core vesicle that contains proteins and/or peptides packaged in the trans-Golgi network and transported to terminals for regulated secretion (7). Paraneurons, such as thyroid parafollicular (PF) cells, are endocrine cells that are closely related to neurons (8). They are derived embryologically from the neural crest (9) and can be induced by nerve growth factor to assume a neuronal phenotype in vitro (10). The secretory vesicles of PF cells share characteristics of both the large trans-Golgi network-derived, peptide-containing dense core vesicles and the small, endosome-derived, low molecular weight neurotransmitter-containing SVs. PF secretory vesicles resemble large, dense core vesicles in their size, initial formation in the trans-Golgi network (11, 12), and content of calcitonin and other peptides (13); however, like SVs, PF secretory vesicles contain a small molecule neurotransmitter, 5-HT (14–16), and they recycle (17). In contrast to the peptides that are added to vesicles at the time of their formation in the trans-Golgi network, 5-HT is loaded into PF vesicles by transmembrane transport from the cytosol (15, 18). As is generally true of monoamines in SVs, this transport is mediated by a transporter protein in the vesicular membrane (19) and driven by a transmembrane proton gradient (18) that is established by the vesicular H+–ATPase (20). SV proteins, including the synaptotagmin I, synaptophysin, and synaptobrevin (1), are also present in the membrane of PF secretory vesicles (13, 48).

PF secretory vesicles exhibit properties that have not yet been detected either in neuronal SVs or large, dense core vesicles. The internal environment of PF secretory vesicles is regulated by the same stimuli that initiate secretion (18, 22, 48). The interior of the secretory vesicles of PF cells becomes acidic only when the cells are stimulated by a secretagogue, such as increased extracellular Ca2† (↑[Ca2+]i). Because the membranes of PF vesicles are not permeable to Cl− under resting conditions, influx of H+ is limited by the generation of a transmembrane potential gradient (ΔΨ) (18, 22). In response to stimulation of PF cells with a secretagogue, a Cl− channel opens in the vesicular membrane (18). The opening of this channel dissipates ΔΨ and permits Cl− to enter vesicles as a counterion (48) so that transport of H+ is no longer electrogenic (18) and acidification of vesicles can proceed (22).

The mechanism responsible for stimulus acidification coupling has not yet been determined. A 64-kDa protein (p64), which is identical to a Cl− channel that has been cloned from epithelial cells (23), is present in the membranes of PF secretory vesicles (48). This protein has potential phosphorylation sites, and stimulation of PF cells with a secretagogue increases the phosphorylation of vesicular p64 (48). Acidification of vesicles, moreover, is inhibited by compounds that antagonize a variety of protein kinases or phosphatases (48). These observations are consistent with the following working hypotheses: (i) p64 is the Cl− channel in vesicular membranes that permits...
the secretion-induced entry of Cl⁻ into vesicles; (ii) the state and site of phosphorylation of p64 determine whether the channel is open or closed; (iii) signal transduction pathways (still to be identified) couple plasmalemmal secretogogue receptors to protein kinases and/or phosphatases that determine the level of phosphorylation of the channel protein. Because stimulus acidification and stimulus secretory coupling can be dissociated (48). The signal transduction pathways responsible for each are not identical.

Because the acidification of secretory vesicles is evoked by exposing PF cells to secretogogues, both acidification and secretion could conceivably be mediated either by a cell surface Ca²⁺ receptor or by a channel that is directly sensitive to the [Ca²⁺]ᵢ. Recently, each of the [Ca²⁺]ᵢ-sensitive endocrine cells, parathyroid chief cells (24), and PF cells (49), have been found to express a plasmalemmal calcium receptor (CaR). The CaR is a heptahedral integral membrane protein that when expressed in oocytes, couples to a pertussis toxin-sensitive G protein (24). Because [Ca²⁺]ᵢ evokes secretion in PF cells and inhibits secretion in parathyroid cells, it is possible that the CaR in each cell type is coupled to a different G protein. The current experiments were undertaken to test the hypothesis that the PF cell CaR is responsible for mediating the effects of [Ca²⁺]ᵢ on vesicle acidification. Our data demonstrate that the acidification of the secretory vesicles in PF cells is mediated via activation of the CaR and that the CaR is coupled via Gᵢ and to a cascade of second messengers, including inositol trisphosphate (IP₃), cytosolic free Ca²⁺, NO, cGMP, and protein kinase G, resulting in the phosphorylation and gating of the Cl⁻ channel in the membranes of PF secretory vesicles.

MATERIALS AND METHODS

Isolation of PF Cells—Fresh sheep thyroid glands were obtained from a local abattoir. The glands were dissociated with trypsin, and PF cells were isolated by “phagocytic chromatography” as described previously (16, 18, 26). This method utilizes thyrotropin to activate the follicular cells, which are induced by thyroid-stimulating hormone to become phagocytic. When a thyroid-stimulating hormone-stimulated suspension of thyroid cells is passed through a column of Sephadex beads coupled to thyroglobulin, the follicular cells in the suspension “attempt” to phagocytize the beads and remain on the surface, whereas the PF cells pass through in the void volume. Red cells are then removed from the suspension by centrifugation through a layer of Ficoll. In the final preparation, 97% of cells are parafollicular; the remainder are mainly fibroblasts, and there are no detectable follicular cells. Purified PF cells are allowed to adhere to glass and are then exposed to CaR agonists and/or experimental conditions followed by the isolation procedure by culture overnight at 37 °C. The culture medium consisted of Eagle’s minimum essential medium supplemented with 10% fetal bovine serum and buffered by CO₂.

Determination of Cytosolic Ca²⁺—Using Fura-2/AM—Levels of cytosolic Ca²⁺ ([Ca²⁺]ᵢ) were measured as described previously (27). Briefly, PF cells (5 × 10⁶ cells/ml) were loaded with fura-2 by incubation for 30 min in a solution containing Ca²⁺-free Hanks’ solution and the ace-toxymethyl ester of fura-2 (fura-2/AM; 5.0 μM). The cells were then washed twice, diluted (to 2 × 10⁶ cells/ml) in the same medium, incubated for an additional 20 min, and washed again. To measure [Ca²⁺]ᵢ, aliquots of the fura-2-loaded cells (2 × 10⁶ cells/ml) were suspended at 25 °C in stirred cuvettes to which experimental compounds were added. The ratio of the intensity of fluorescence (measured at 505 nm) of cells excited with light at 340 nm to that of cells excited with light at 380 nm was determined and used to estimate [Ca²⁺]ᵢ (28).

Acidification of PF Vesicles—The weak base, acridine orange (AO), becomes trapped and thus concentrated in acidic compartments within cells. The fluorescence of AO is quenched by a red shift in the fluorescence of AO, which is a function of the AO concentration in vesicles. The cytoplasm of PF cells contains so many secretory vesicles that the AO fluorescence of whole cells is essentially that of their vesicles (22). AO trapping was used to evaluate the acidification of PF secretory vesicles as described previously (48). Purified PF cells were incubated with AO (6.0 μM) for 5 min at 37 °C and then exposed to CaR agonists and/or experimental compounds for 5–15 min at 37 °C. Cells were preincubated with potential inhibitors for 10 min before loading with AO. AO fluorescence was detected with a Zeiss microscope equipped with a vertical illuminator and a 40× objective (0.9 numerical aperture). Fluorescence was elicited with light at 450–490 nm and passed through a long pass band emission filter that enabled both green and red fluorescence to be visualized simultaneously. Up to 500 cells were examined for each set of conditions, scored as red (acidic) or green (nonacidic), and the percentage of each was calculated.

Immunocytochemical Detection of NO Synthase—Isolated PF cells, cultured on glass coverslips as monolayers were fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate-buffered saline (pH 7.4). The cells were then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 30 min and exposed overnight to polyclonal antibodies to NO synthase (diluted 1:250), which were localized with goat anti-rabbit IgG coupled to horseradish peroxidase. Peroxidase activity was visualized with 3,3′-diaminobenzidine and H₂O₂. In control experiments, the primary antibodies were omitted, and nonimmune sera were substituted.

[³⁵S]GTP·γ·S Binding to G Proteins and Immunoprecipitation—Identification of G Proteins activated by stimulation of the CaR was studied by determining which G proteins were induced by stimulation of the receptor to bind GTP (30). Essentially, a membrane fraction is incubated with an agonist, [³⁵S]GTP·γ·S, in the presence of a P₃-labeled nonhydrolyzable analog of GTP. The radioactive α subunits of activated G proteins are then immunoprecipitated with monospecific antibodies and identified by Western blotting. PF cells (2 × 10⁶) were homogenized in 2 ml of buffer, and a crude membrane fraction was prepared by centrifugation. The pellet was resuspended in 2 ml of homogenization buffer, and 1 ml was incubated with 20 μl of antibody (10% of 20% protein A-Sepharose CL-4B). The suspension was centrifuged, and the α subunits of specific G proteins were immunoprecipitated from the supernatant. Antibodies that were employed were anti-Gα₁i, anti-Gα₁q, and anti-Gα₅ (react with all known α subunits). All antibodies were diluted 1:1000 and were incubated with membranes in 200 μl of solution at 30 °C for 30 min. Protein A-Sepharose CL-4B (100 μl; 100% α) was then added to enhance the immunoprecipitation of the immune complex, and the incubation was continued for an additional 30 min. The suspension was centrifuged, the pellets were washed and resuspended in Kreb’s-Ringer solution, and the radioactivity was determined by liquid scintillation.

The specific binding was determined by subtracting the amount precipitated by a 1:1000 dilution of normal rabbit serum from that precipitated with the specific antibody.

Pertussis Toxin—In order to examine the effects of PTx on responses of cultured PF cells to CaR agonists, PTx was activated by preincubation for 30 min at a concentration of 50 μg/ml in 50 ml Hepes buffer (pH 8.0) containing bovine serum albumin (1.0 mg/ml), dithiothreitol (20 mM), and SDS (0.1%). Cultures were incubated at 37 °C with activated PTx (500 ng/ml) for 90 min. Acidification of vesicles was then investigated in control and PTx-treated cells in response to a test stimulus provided by application of Ca²⁺ (1.0 mM) or Gd³⁺ (0.25 mM).

In order to test the effect of PTx on the binding of [³⁵S]GTP·γ·S in response to stimulation by ligand, membrane fraction obtained from PF cells (50 μg in 300 μl) was preincubated with activated PTx (500 ng/ml) for 30 min at 37 °C. Membranes were then washed and incubated with 2.0 nM [³⁵S]GTP·γ·S. Immunoprecipitation of the G₁ subunits was then investigated in control and PTx-treated fractions.

Drugs and Chemicals—All drugs and chemicals were obtained from Sigma, unless otherwise specified. The L-NAME (Nω-nitro-L-arginine methyl ester), a Nω-channel blocker, n motifetine, the P-type voltage-gated Ca²⁺ channel blocker, 8-g antagonist IVA, the N-type voltage-gated Ca²⁺ channel blocker, Nω-conotoxin GVIA, and thapsigargin, which inhibits pumps of the endoplasmic reticulum, were purchased from Alomone Labs. (Jerusalem, Israel). The PCK inhibitors, staurosporine, calphostin C, and chelerythrine, were obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). LY-83583, an inhibitor of guanylyl cyclase, was purchased from Biomol Research Labs. (Plymouth Meeting, PA). L-N⁵(1-iminoethyl)ornithine hydrochloride (imino-ornithine), which inhibits NO synthase, was obtained from

The culture medium consisted of Eagle’s minimum essential medium supplemented with 10% fetal bovine serum and buffered by CO₂.
Calcium-induced Gated Vesicular Chloride Channels

RBI (Natick, MA). Rp-B-(4-chlorophenylthio)-GMP S, a membrane-permeant inhibitor of protein kinase G, was purchased from Biolog Life Science Institute (La Jolla, CA). [35S]GTPγS and antibodies to the α subunits of G proteins were purchased from DuPont NEN. Protein A-Sepharose CL-4B was obtained from Pharmacia Biotech Inc. Polyubiquitins of G proteins were purchased from DuPont NEN. Protein Science Institute (La Jolla, CA). [35S]GTP was purchased from Biolog Life Science Institute (La Jolla, CA). Rp-8-(4-chlorophenylthio)-cGMPS, a membrane-permeant inhibitor of protein kinase G, was purchased from Biolog Life Science Institute (La Jolla, CA).

RESULTS

The CaR Agonists, Ca2+ and Gd3+, Cause the Secretory Vesicles of PF Cells to Acidify—Acidification of secretory vesicles was assayed microscopically by detecting the trapping of AO (22). The addition of either Gd3+ or Ca2+ to the media in which PF cells were suspended induced the vesicles of these cells to acidify. The response to each agonist was concentration-dependent (Fig. 1A). In the absence of a calcium channel blocker, the response to Ca2+ did not saturate in the range of concentrations examined (Fig. 1A); however, saturation was seen when PF cells were challenged with Ca2+ in the presence of the L-type calcium channel blocker, nimodipine (10 μM) (Fig. 1A). No additional inhibition was seen when the N-type channel blocker, ω-conotoxin, and the P-type channel blocker, agatoxin, were added to nimodipine-containing media (data not illustrated). These observations suggest that Ca2+ at low concentrations induces vesicle acidification by a mechanism that is independent of calcium channels; however, the increment in acidification that occurs at concentrations of Ca2+ above 1-2 mM is probably dependent on L-type calcium channels. In contrast to the Ca2+ response, the acidification of vesicles induced by Gd3+, which is itself a calcium channel blocker (31), saturated at concentrations above 100 μM (Fig. 1B). The maximal degree of acidification induced by Gd3+, which was virtually identical to that of the calcium channel-independent component (measured in the presence of nimodipine) of the response to Ca2+ (Table I). The concentration effect curves for Gd3+ and Ca2+ in the presence of nimodipine were parallel, but Gd3+ (ED50 = 52 μM) was a more potent stimulator of acidification than was Ca2+ (ED50 = 636 μM; in the presence of nimodipine) (Table I). Acidification of vesicles in response to 250 μM Gd3+ or to 1.0 mM Ca2+ in the presence of nimodipine was abolished (reduced by 95 ± 3%) by PTx (p < 0.01 versus control; n = 5). These observations are compatible with the idea that Gd3+ and Ca2+ acidify vesicles through an action at a receptor that is coupled to a PTX-sensitive G protein. Stimulation of PF Cells by the CaR Agonists, Ca2+ or Gd3+, Induces the Binding of [35S]GTPγS to Gαi and Gαβγ-Because the CaR is a heptahelical receptor (24, 49), it is presumably coupled to a G protein. Although there is evidence that the CaR couples to a PTX-sensitive G protein in transfected oocytes, that protein has not been identified. G protein coupling of the endogenous CaR in PF cells has not previously been investigated, although the PTX sensitivity of CaR agonist-induced acidification suggests that the receptor might be coupled to a G protein of the G/Gi class, which are PTX-sensitive. We therefore investigated the coupling of the CaR to G proteins in PF cells.

PF cell membranes were isolated and stimulated with Ca2+ or Gd3+. Isolated PF cell membranes were incubated with [35S]GTPγS and exposed to Ca2+ (1 mM) or Gd3+ (250 μM). The radioactivity immunoprecipitated by antibodies that react specifically with Gαi, Gαq, or all known Gα subunits was determined. Baseline values were also ascertained for control preparations, in which membranes were incubated without agonists and in the presence of EGTA. The data were expressed as the ratio of the radioactivity immunoprecipitated from membranes incubated in the presence of agonists to that of the corresponding controls in the same experiment. The effectiveness and specificity of the antibodies were evaluated by immunoblotting (30) (Fig. 2). The antibodies we used for immunoprecipitation (anti-Gαi and anti-Gαq) were specific in that anti-Gαi immunoprecipitated Gαi but not Gαq and anti-Gαq immunoprecipitated Gαq but not Gαi. Antibodies to the membrane Cl– channel, p64, failed to immunoprecipitate either Gαi or Gαq. Both
Ca\(^{2+}\) and Gd\(^{3+}\) were found to stimulate the binding of \([^{35}\text{S}]\text{GTP-}\gamma\text{S}\) to \(G_{\alpha i}\), and, to a much lesser extent, also to \(G_{\alpha q}\) (Fig. 3A and B) \((p < 0.01\) for Gd\(^{3+}\); \(p < 0.05\) for Ca\(^{2+}\) versus control). The concentrations of the agonists used in these experiments were each supramaximal with respect to the acidification of vesicles (see Fig. 1B). At these concentrations, the degree of stimulation of \([^{35}\text{S}]\text{GTP-}\gamma\text{S}\) binding to \(G_{\alpha i}\) and \(G_{\alpha q}\) evoked by Ca\(^{2+}\) was not significantly different from that evoked by Gd\(^{3+}\).

The sum of the radioactivity immunoprecipitated by antibodies to \(G_{\alpha i}\) and \(G_{\alpha q}\) was equal to that immunoprecipitated by antibodies that react with all known \(G\) subunits, suggesting that no additional \(G\) proteins are stimulated by Ca\(^{2+}\) or Gd\(^{3+}\). When membranes were treated with PTx, the Ca\(^{2+}\)-induced binding of \([^{35}\text{S}]\text{GTP-}\gamma\text{S}\) to \(G_{\alpha i}\) was abolished (Fig. 3B); however, the stimulation by Ca\(^{2+}\) of the binding of \([^{35}\text{S}]\text{GTP-}\gamma\text{S}\) to \(G_{\alpha q}\) was not affected by PTx (Fig. 3B).

The CaR Agonists, Ca\(^{2+}\) and Gd\(^{3+}\), Mobilize Ca\(^{2+}\) from an Internal Source—Isolated PF cells were loaded with fura-2 in order to measure the concentration of cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_c\)). Exposure of the PF cells to Gd\(^{3+}\) evoked a concentration-dependent increase in [Ca\(^{2+}\)]\(_c\) with an ED\(_{50}\) of 40 \(\mu\text{M}\) (Fig. 4). This increase was unaffected by the addition of 10 \(\mu\text{M}\) nimodipine (data not illustrated). Because Ca\(^{2+}\) could not have entered cells from the outside under these conditions, it can be concluded that the external application of Gd\(^{3+}\) leads to the release of Ca\(^{2+}\) from an internal source. Similar experiments were conducted with Ca\(^{2+}\). Again, as occurred when PF cells were incubated with Gd\(^{3+}\), exposure of PF cells to \(\text{[Ca}^{2+}]\_c\) induced an increase in [Ca\(^{2+}\)]\(_c\) (Fig. 5). In this case, however, the ability of Ca\(^{2+}\) to enter cells via calcium channels means that extracellular Ca\(^{2+}\) is a potential source of the increased [Ca\(^{2+}\)]. The L-type calcium channel blocker, nimodipine, did not prevent the rise in [Ca\(^{2+}\)]\(_c\) induced by exposure of PF to Ca\(^{2+}\); until concentrations of [Ca\(^{2+}\)]\(_c\) in excess of 1–2 \(\mu\text{M}\) were reached. At higher concentrations of [Ca\(^{2+}\)]\(_c\), the continued rise in [Ca\(^{2+}\)]\(_c\) was blocked by nimodipine. These data suggest that low concentrations of [Ca\(^{2+}\)]\(_c\) liberate Ca\(^{2+}\) from an internal source, but that entry of Ca\(^{2+}\) through nimodipine-sensitive calcium channels contributes to the increase in [Ca\(^{2+}\)]\(_c\) that occurs at concentrations above 1–2 \(\mu\text{M}\). The effectiveness of low concentrations of [Ca\(^{2+}\)]\(_c\) in inducing an increase in [Ca\(^{2+}\)], appeared to be enhanced in the presence of nimodipine, suggesting that the sensitivity of the CaR may increase when L-type calcium channels are blocked. The change in [Ca\(^{2+}\)]\(_c\), evoked by exposing PF cells to increasing concentrations of [Ca\(^{2+}\)]\(_c\), was not further affected by adding \(\omega\)-agatoxin and a-gatoxin (data not illustrated) to nimodipine-containing media.

The increase in [Ca\(^{2+}\)]\(_c\) participates in CaR-induced Vesicle Acidification—Thapsigargin causes the release and ultimately the depletion of IP\(_3\)-releasable internal Ca\(^{2+}\) stores (32). 10 min after exposure of PF cells to thapsigargin (10 \(\mu\text{M}\), vesicles acidified (to 135 \(\pm\) 5\% of control; \(n = 4\)). This effect was transient and was no longer present after 60 min of incubation in the continued presence of thapsigargin. At this time, the level of [Ca\(^{2+}\)]\(_c\), was lower than control in the thapsigargin-treated cells, and challenge with Gd\(^{3+}\) (250 \(\mu\text{M}\)) no longer evoked an increase in [Ca\(^{2+}\)]\(_c\) (Fig. 6A). Exposure of thapsigargin-treated cells to low concentrations of [Ca\(^{2+}\)]\(_c\), even in the presence of nimodipine, caused the [Ca\(^{2+}\)]\(_c\) to rise (Fig. 6B); nevertheless, the level of [Ca\(^{2+}\)]\(_c\), reached following a challenge with [Ca\(^{2+}\)]\(_c\), was less in the thapsigargin-treated cells than in controls. These data suggest that some Ca\(^{2+}\) may enter PF cells from the external medium via a route that does not involve L-type calcium channels, at least when internal Ca\(^{2+}\) has been depleted by incubation with thapsigargin. In addition to its effect on [Ca\(^{2+}\)]\(_c\), thapsigargin blocked the acidification of vesicles induced by Gd\(^{3+}\) and inhibited that induced by Ca\(^{2+}\) (Fig. 6C). These data suggest that the release of Ca\(^{2+}\) from a thapsigargin-sensitive internal compartment is a critical compo-

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**Fig. 2.** Western blots showing the specificity of antibodies used to immunoprecipitate \(G_{\alpha i}\) and \(G_{\alpha q}\). Lane 1, immunoprecipitate obtained with anti-Gao and probed with antibodies to \(G_{\alpha q}\); lane 2, immunoprecipitate obtained with anti-Gao and probed with antibodies to \(G_{\alpha i}\); lane 3, immunoprecipitate obtained with anti-Gal and probed with antibodies to \(G_{\alpha i}\); lane 4, immunoprecipitate obtained with anti-p64 (membrane Cl\(^{-}\) channel) and probed with antibodies to \(G_{\alpha q}\).

**Fig. 3.** Gd\(^{3+}\) and [Ca\(^{2+}\)]\(_c\), activate \(G_{\alpha i}\) and \(G_{\alpha q}\). Membranes isolated from PF cells were incubated with Gd\(^{3+}\) (250 \(\mu\text{M}\)) or Ca\(^{2+}\) (1.0 \(\mu\text{M}\)) in the presence of \([^{35}\text{S}]\text{GTP-}\gamma\text{S}\). A, radioactive \(G_{\alpha i}\) and \(G_{\alpha q}\) were immunoprecipitated from membranes stimulated with Gd\(^{3+}\). The data are expressed as the experimental to control (no agonist) ratio, so that a value of 1.00 (dotted line) indicates no effect. Note that far more \([^{35}\text{S}]\text{G}_{\alpha i}\) than \([^{35}\text{S}]\text{G}_{\alpha q}\) was immunoprecipitated. B, \([^{35}\text{S}]\text{G}_{\alpha i}\) and \([^{35}\text{S}]\text{G}_{\alpha q}\) were immunoprecipitated from membranes stimulated with Ca\(^{2+}\). Again, as with membranes stimulated by Gd\(^{3+}\), \([^{35}\text{S}]\text{G}_{\alpha i}\) >> \([^{35}\text{S}]\text{G}_{\alpha q}\). The activation of \(G_{\alpha i}\), but not that of \(G_{\alpha q}\), was prevented by preincubation of membranes with PTx. The experimental to control ratio for \(G_{\alpha q}\) stimulation by Ca\(^{2+}\) was 1.26, which is a very small change but significantly different from 1.00 \((t = 2.479; p < 0.05)\). 1590 \(\pm\) 250 cpm were obtained in controls; following activation, the radioactivity associated with the same amount of protein of \(G_{\alpha q}\) was 6757 \(\pm\) 1050 and 5962 \(\pm\) 925 cpm for Gd\(^{3+}\) and Ca\(^{2+}\), respectively. The radioactivity associated with \(G_{\alpha q}\) following activation was 2170 \(\pm\) 100 and 2105 \(\pm\) 50 cpm for Gd\(^{3+}\) and Ca\(^{2+}\), respectively.
ment of stimulus acidification coupling for responses to both Gd\(^{3+}\) and Ca\(^{2+}\).

Neither ryanodine (10 \(\mu\)M) nor caffeine (10 mM) increased \([Ca^{2+}]_i\) or acidified the vesicles of PF cells (data not illustrated). These observations suggest that PF cells lack ryanodine receptors and are consistent with the idea that the CaR mobilizes Ca\(^{2+}\) from an IP\(_3\)-sensitive source. This hypothesis was tested. The CaR was stimulated with either Gd\(^{3+}\) (250 \(\mu\)M) or a low concentration of Ca\(^{2+}\) (1.0 mM), vesicle acidification was measured, and the ability of U 73122, a specific inhibitor of PI-phospholipase C, to antagonize the response was determined. U 73122 (10 \(\mu\)M) but not U 73343 (10 \(\mu\)M), an inactive congener of U 73122, blocked the Gd\(^{3+}\)-induced acidification of PF vesicles (Table II). U 73122 also inhibited the Gd\(^{3+}\)-induced increase in \([Ca^{2+}]_i\) (Table II). These data confirm that PI-phospholipase C and thus IP\(_3\) mediate the increase in \([Ca^{2+}]_i\) that follows stimulation of the CaR; moreover, the observations

\[\text{Table II}
\begin{array}{ccc}
\hline
\text{Addition} & \text{[Ca}^{2+}]_i \text{, M} & \text{\% control} \\
\hline
\text{Gd}^{3+} & 150 \pm 9 & 145 \pm 14 \\
\text{Gd}^{3+} + \text{U73122} & 101 \pm 1^a & 103 \pm 2^a \\
\text{Gd}^{3+} + \text{U73343} & 147 \pm 7^b & 136 \pm 11 \\
\hline
\end{array}
\]

\(^a p < 0.005\) versus Gd\(^{3+}\) alone.  
\(^b p < 0.005\) versus Gd\(^{3+}\) + U73122; \(n = 3\) for all groups.
NO synthase immunoreactivity can be detected in PF cells by immunocytochemistry. A, isolated PF cells show NO synthase immunoreactivity. B, PF cells show no immunostaining when antibodies to NO synthase are omitted and nonimmune serum is substituted. Bars, 15 μm.

Fig. 7. NO synthase immunoreactivity can be detected in PF cells by immunocytochemistry. A, isolated PF cells show NO synthase immunoreactivity. B, PF cells show no immunostaining when antibodies to NO synthase are omitted and nonimmune serum is substituted. Bars, 15 μm.

Fig. 8. NO appears to participate in signaling for Gd3+ and [Ca2+]i-induced vesicle acidification. A, Gd3+, [Ca2+]i, the NO donor, sodium nitroprusside (1.0 mM), and dibutyryl cGMP (1.0 mM) all induce vesicle acidification. Imino-ornithine (10 μM), a NO synthase inhibitor, blocks the response to Gd3+ and [Ca2+]i, but not to sodium nitroprusside or dibutyryl cGMP. Shaded bars, no inhibitor; bars with dots, imino-ornithine. B, LY 83583 (10 μM), a guanyl cyclase inhibitor, antagonizes vesicle acidification in response to Gd3+, [Ca2+]i, and sodium nitroprusside but does not affect the response to dibutyryl cGMP. Rp-cGMPS (10 μM), an inhibitor of protein kinase G, antagonizes vesicle acidification induced by Gd3+ and [Ca2+]i. Lightly shaded bars, no inhibitor; hatched bars, LY 83583; darkly shaded bars, Rp-8(4-chlorophenylthio)-cGMPS.

also provide additional support for the idea that the rise in [Ca2+]i is a critical step in the signal transduction pathway that leads to vesicle acidification.

NO and cGMP Participate in CaR Signal Transduction—Immunocytochemical studies demonstrated that the constitutive form of NO synthase is present in sheep PF cells (Fig. 7), which also contain histochemically demonstrable NADPH diaphorase activity (data not shown). NO synthase has been shown to be an NADPH diaphorase (33). Because [Ca2+]i is increased when the CaR is stimulated and NO synthase is known to be activated by Ca2+/calmodulin (34), we tested the hypothesis that NO is involved in the mediation of CaR-induced vesicle acidification. The specific NO synthase inhibitor, imino-ornithine (10 μM) (35) was found to block acidification of vesicles in response to either Gd3+ or to Ca2+ (Fig. 8A); moreover, sodium nitroprusside (1.0 mM), a generator of NO (34), caused PF vesicles to acidify (Fig. 8A). The action of sodium nitroprusside, furthermore, was mimicked by dibutyryl cGMP (1.0 mM) (Fig. 8B). This observation is consistent with the idea that NO participates in the acidification signal transduction pathway because guanylyl cyclase is commonly activated by the CaR, Gd3+, and Ca2+, this NO synthase inhibitor did not affect responses to sodium nitroprusside or dibutyryl cGMP (Fig. 8B), suggesting that imino-ornithine does indeed act specifically to prevent the generation of NO and not to inhibit its action. Neither sodium nitroprusside nor dibutyryl cGMP affected the [Ca2+]i (data not shown), suggesting that these agents act downstream from the increase in [Ca2+]i in the signal transduction pathway.

PKC Promotes Vesicle Acidification—The idea that the CaR activates PI-phospholipase C to generate IP3 implies that diacyl glycerol is also produced as a result of stimulation of the CaR. Because diacyl glycerol is an activator of PKC, it is possible that PKC, as well as a G-kinase, regulates the vesicular Cl− conductance that is responsible for vesicle acidification. We thus tested the hypothesis that PKC plays a role in acidification of PF vesicles. Treatment of PF cells with the PKC activator, PMA (10 nM; 10 min), did not significantly change [Ca2+]i (control = 156 ± 14 nM; PMA = 130 ± 13 nM; n = 5) but caused vesicles to acidify (Fig. 9A). The acidification induced by PMA was unaffected by imino-ornithine and LY 83583 (data not illustrated), but it was blocked by staurosporine (10 nM) (Fig. 9A). Staurosporine, however, did not inhibit the acidification of vesicles induced by either Gd3+ (250 μM) or Ca2+ (1.0 mM) (Fig. 9A). Furthermore, neither more specific inhibitors of PKC, chelerythrine (0.5 μM) and calphostin C (1.0 μM) (data not illustrated), nor the down-regulation of PKC by overnight exposure to PMA (10 nM; Fig. 9A) were able to affect Gd3+-induced vesicle acidification. These observations indicate that...
Although PKC can, when activated, cause PF vesicles to acidify, the enzyme is not likely to be involved in transducing CaR-related vesicle acidification.

Because studies with nimodipine indicated that an increment in vesicle acidification above that induced by the CaR occurred when Ca$^{2+}$ entered cells through L-type calcium channels (see Fig. 1A), we tested the possibility that PKC contributes to the Ca$^{2+}$-related acidification of vesicles. PF cells were exposed to 5.0 mM [Ca$^{2+}$]$\text{_{i}}$ (rather than 1.0 mM) in the absence of nimodipine, so that Ca$^{2+}$ would enter cells through calcium channels. When this was done, vesicles acidified strongly (Fig. 9B). Both staurosporine and down-regulation of PKC now significantly antagonized vesicle acidification (Fig. 9B); however, neither of these inhibitory treatments totally prevented acidification of vesicles. Instead, the level of acidification induced by 5.0 mM Ca$^{2+}$ in the presence of staurosporine or after PKC down-regulation was reduced to a level that approximated that induced by 1.0 mM Ca$^{2+}$ (Fig. 9B). These data are consistent with the idea that although PKC does not mediate the basal acidification of vesicles induced by the CaR, it is responsible for the Ca$^{2+}$-channel-related increment in vesicle acidification.

Activation of the CaR by Ba$^{2+}$ Leads to Vesicle Acidification—Ba$^{2+}$ is known to bind to the CaR and to stimulate the CaR expressed in the plasma membranes of transfected oocytes (24). In contrast, concentrations of Ba$^{2+}$ that cause PF cells to secrete have been found to fail to induce vesicle acidification (48). This apparent discrepancy could be explained if Ba$^{2+}$ were to enter cells and exert an intracellular effect that inhibits CaR signal transduction. Ba$^{2+}$ is known to be able to enter cells through calcium channels and to act on intracellular targets to release internal Ca$^{2+}$ and block Ca$^{2+}$ efflux (38). In previous study we have failed to acidify vesicles by activating CaR with Ba$^{2+}$. In confirmation of the previous observation, vesicles were not found to acidify when PF cells were exposed only to Ba$^{2+}$ (Fig. 10A). In contrast, when Ba$^{2+}$ entry into the cells through L-type calcium channels was blocked with nimodipine (10.0 $\mu$m), Ba$^{2+}$ effectively induced vesicle acidification (Fig. 10A). Ba$^{2+}$ also increases [Ca$^{2+}$]$\text{_{i}}$ (by 2.2 $\pm$ 0.2-fold), even in the presence of nimodipine, $\omega$ conotoxin, and agatoxin to block voltage-gated calcium channels. These observations are compatible with the idea that Ba$^{2+}$ can stimulate the CaR, but that this action is negated by an intracellular action of Ba$^{2+}$ if Ba$^{2+}$ can enter the cells. If that idea is correct, then Ba$^{2+}$ would be expected to inhibit vesicle acidification in response to CaR stimulation by Ca$^{2+}$ but not that induced by Gd$^{3+}$. When Ca$^{2+}$ is the agonist, nothing would prevent Ba$^{2+}$ entry, and the intracellular inhibitory effect of Ba$^{2+}$ would be manifested. In contrast, when Gd$^{3+}$ is the CaR agonist, Ba$^{2+}$ would be prevented from entering the cells because Gd$^{3+}$ is also a calcium channel blocker. In this case, the intracellular action of Ba$^{2+}$ would be prevented, and only its effect on the CaR would be manifest. These predictions were confirmed. Ba$^{2+}$ blocked the acidification of vesicles induced by Ca$^{2+}$ (5.0 mM) but did not antagonize that induced by Gd$^{3+}$ (250 $\mu$m). In fact, Ba$^{2+}$ enhanced the action of Gd$^{3+}$, suggesting that Ba$^{2+}$ like Gd$^{3+}$ is a CaR agonist when Ba$^{2+}$ is prevented from entering cells through calcium channels and that the effects of Ba$^{2+}$ and Gd$^{3+}$ are additive (Fig. 10B).

**DISCUSSION**

The current study was undertaken to determine whether the CaR is responsible for the phenomenon of secretagogue-evoked acidification of secretory vesicles in PF cells and if so, to characterize the signal transduction pathway. It was, however, first necessary to distinguish effects mediated by the CaR from those resulting from the entry of Ca$^{2+}$ through plasma membrane calcium channels. Gd$^{3+}$ was particularly valuable for this purpose. Gd$^{3+}$ is both an efficient ligand at the CaR (8, 24) and a highly effective calcium channel blocker (39); therefore, when Gd$^{3+}$ is used to activate the CaR, responses are not complicated by the influx of Ca$^{2+}$ through calcium channels. Gd$^{3+}$ was found to be an agonist that induced vesicle acidification in a concentration-dependent manner. A similar, but more complicated response was elicited by [Ca$^{2+}$]$\text{_{i}}$. At low concentrations of [Ca$^{2+}$]$\text{_{i}}$ ($\leq$ 1.0 mM), vesicle acidification was unaffected by the addition of calcium channel blockers and the concentration effect curve was roughly parallel to that of Gd$^{3+}$, although it was shifted to the right. These data suggest that vesicle acidification in response to low concentrations of [Ca$^{2+}$]$\text{_{i}}$, and Gd$^{3+}$: is the result of an action at a common receptor and that Gd$^{3+}$ is a more potent agonist. At higher concentrations of [Ca$^{2+}$]$\text{_{i}}$, however, an increment in acidification occurred that was beyond that induced by Gd$^{3+}$. This increment was blocked by the
L-type calcium channel blocker, nimodipine. It is possible that this increment represents the influx of Ca\(^{2+}\) in a subpopulation of cells that does not respond to low concentrations of Ca\(^{2+}\). These observations suggest that acidification of PF vesicles can be induced by mechanisms that are both independent of and dependent on plasma membrane calcium channels.

A third CaR agonist, Ba\(^{2+}\), provided further insight into the nature of calcium channel-independent vesicle acidification. By itself, Ba\(^{2+}\) causes PF cells to secrete, but it does not induce vesicle acidification (48). This observation provided the initial evidence that acidification of vesicles is not a requirement for secretion by PF cells and that the transduction mechanisms responsible for vesicle acidification and secretion are not identical. When added together with nimodipine, however, Ba\(^{2+}\) caused vesicles to acidify; moreover, the response to Ba\(^{2+}\) was synergistic with that of Gd\(^{3+}\). In contrast, in the absence of nimodipine, Ba\(^{2+}\) inhibited the vesicle acidification induced by low concentrations of Ca\(^{2+}\). These observations suggest that Ba\(^{2+}\), like Gd\(^{3+}\) and Ca\(^{2+}\), can cause vesicles to acidify through a mechanism that is independent of calcium channels. This effect of Ba\(^{2+}\), however, is only manifested when Ba\(^{2+}\) is prevented from entering PF cells. If Ba\(^{2+}\) enters cells, as it does in the absence of calcium, channel blockade can evidently inhibit signal transduction from a plasmalemmal receptor. This intracellular action accounts for the ability of Ba\(^{2+}\) to inhibit the action of Ca\(^{2+}\) when Ba\(^{2+}\) is allowed to enter PF cells. In the absence of nimodipine, therefore, the plasmalemmal action of Ba\(^{2+}\) is probably masked by a countering intracellular effect. The intracellular inhibitory action of Ba\(^{2+}\), however, is not manifest when Ba\(^{2+}\) is applied together with Gd\(^{3+}\), which prevents the entry of Ba\(^{2+}\) through calcium channels. The observation that each of three CaR agonists, Ca\(^{2+}\), Gd\(^{3+}\), and Ba\(^{2+}\) can induce vesicle acidification by a plasmalemmal action that is independent of calcium channels, supports the idea that the CaR is responsible for their common effect. The nimodipine-dependent increment in vesicle acidification induced by concentrations of Ca\(^{2+}\) above 1.0 mM suggests that Ca\(^{2+}\) entry through L-type calcium channels can also contribute to vesicle acidification through a process that does not involve the CaR.

If the CaR is responsible for agonist-induced vesicle acidification, then stimulus-acidification coupling would be expected to be mediated by a signal transduction pathway that involves a G protein (8, 24). The identification of such a pathway would thus simultaneously provide evidence for a role of the CaR in vesicle acidification and also provide important insight into the responsible mechanism. We thus tested the roles played by G proteins, likely second messengers, and effectors in mediating vesicle acidification in response to CaR agonists (Gd\(^{3+}\) or a low concentration of Ca\(^{2+}\)) in the presence of nimodipine. Vesicle acidification induced by Gd\(^{3+}\) and Ca\(^{2+}\) was abolished by PTx, and these agonists also activated Gi (as determined by measuring the binding of [35S]GTP-G to immunoprecipitated G\(_i\)). Although the agonists were also found to activate G\(_i\), this effect was not, like that of the activation of G\(_i\), inhibited by PTx. The ability of Ca\(^{2+}\) to activate Gq was slight but significant (p < 0.05 when each sample was compared with its own control). It thus seems likely that coupling of the CaR to Gi is a critical step in stimulus acidification coupling. The coupling of the CaR to Gi in PF cells is similar to the coupling of the receptor expressed in oocytes (24). Coupling of the CaR to Gi may be involved in other responses of PF cells to stimulation of the receptor. Stimulus-induced acidification, for example, is distinct from stimulus-induced secretion, which is resistant to PTx (48).

Several observations supported the idea that the G protein activated by the CaR stimulates PI-phospholipase C. Both Gd\(^{3+}\) and low concentrations of Ca\(^{2+}\) mobilized Ca\(^{2+}\) from an internal pool that was sensitive to thapsigargin. Thapsigargin inhibits the calcium pumps of the ER, preventing the reuptake of Ca\(^{2+}\), which leads to an irreversible depletion of IP\(_3\)-mobilizable internal Ca\(^{2+}\) stores (32). Because ryanodine did not mobilize Ca\(^{2+}\), PF cells probably lack ryanodine receptors; therefore, cyclic ADP-ribose, which acts through these receptors (40), is not likely to be involved in the CaR-stimulated mobilization of Ca\(^{2+}\) from internal stores. More direct evidence for the participation of PI-phospholipase C was obtained with the PI-phospholipase C inhibitor, U 73122 (4), which specifically blocked both the mobilization of Ca\(^{2+}\) and the acidification of vesicles in response to Gd\(^{3+}\) or Ca\(^{2+}\). These data suggest that PI-phospholipase C activated secondary to the coupling of the CaR to Gi causes the release of IP\(_3\), which mobilizes Ca\(^{2+}\). Although the ability of the \( \alpha \) subunits of the G\(_i\) family of G proteins to activate PI-phospholipase C has been demonstrated directly (3), it has been difficult to demonstrate PI-phospholipase C activation by the \( \alpha \) subunits of PTX-sensitive G proteins (5, 41, 42); nevertheless, receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate is blocked.
by PTx in many different types of cell (2, 41, 43). Many reports have indicated that the βγ subunits, rather than the α subunits, are the components of PTx-sensitive G proteins, including Gai, which activate PI-phospholipase C (41, 42, 44). It is thus possible that the βγ subunits released from Gai following its coupling to the CaR are responsible for the CaR-dependent activation of PI-phospholipase C. A role for Gai, however, cannot be ruled out. Both the α and the βγ subunits of Gai have been found to participate in the adenosine A1 receptor-mediated activation of PI-phospholipase C (45).

Thapsigargin not only antagonized the agonist-induced increase in [Ca2+]i, but also antagonized stimulus acidification coupling, suggesting that the PI-phospholipase C-mediated increase in [Ca2+]i is a critical component of the transduction pathway. It seems likely that the effect of the mobilization of [Ca2+]i is mediated by activation of NO synthase. PF cells were found by immunocytochemistry to contain NO synthase (and NADPH diaphorase activity), which is known to be activated by Ca2+/calmodulin (46); moreover, the NO synthase inhibitor, imino-ornithine, blocked the vesicle acidification induced by Gd3+ or Ca2+ but not that induced by the NO generator, sodium nitroprusside. In addition, as expected for an effect mediated by NO synthase (28), Gd3+ and Ca2+-induced vesicle acidification were antagonized by an inhibitor of guanylyl cyclase (LY 83583, which also blocked the effects of sodium nitroprusside) and G kinase (Rp-cGMPS). The involvement of NO synthase in stimulus acidification could account for the previous observation that inhibition of calcineurin, a Ca2+/calmodulin-dependent protein phosphatase, antagonizes Ca2+-stimulated vesicle acidification (48). Phosphorylated NO synthase is a substrate for calcineurin, and phosphorylation of NO synthase decreases its catalytic activity (47). The proposed signal transduction pathway for CaR-induced vesicle acidification is shown in Fig. 11.

Although the evidence outlined above supports the hypothesis that the CaR is coupled to vesicle acidification, stimulation of this receptor is not the only means by which vesicle acidification is controlled. It is probable that the entry of Ca2+ through nimodipine-sensitive L-type calcium channels also causes vesicles to acidify, that this effect is independent of the CaR, and that the Ca2+ channel-related and CaR-induced components of vesicle acidification are additive. Thus, a nimodipine-inhibitable increment in acidification is seen when Ca2+ is present at concentrations above that needed to stimulate the CaR. The magnitude of the CaR-mediated component of acidification can be estimated either from the maximal response to Gd3+ or from that to Ca2+ in the presence of nimodipine, which is about the same. The observation that the calcium channel-related component of vesicle acidification, but not that which appears to be stimulated by the CaR, is blocked by inhibitors of PKC (staurosporine and calphostin C) and by PKC down-regulation confirms that independent mechanisms are responsible for the two components. The diacyl glycerol that is generated by the action of PI-phospholipase C activated in response to CaR stimulation could activate PKC. Diacyl glycerol activation, however, seems to be insufficient for vesicle acidification. The data are consistent with the idea that the component of vesicle acidification related to calcium channels is mediated by PKC, whereas that related to the CaR is mediated by a G kinase. In both cases, phosphorylation of p64, the chloride channel in the membranes of PF vesicles could be responsible for increasing the Cl− conductance that underlies acidification (48).

The role played by the CaR in normal homeostasis remains to be determined. [Ca2+]i was observed to activate the receptor in concentrations that are below those normally found in extracellular fluid. The effect of such low concentrations of [Ca2+]i, however, were only detected in comparison to cells incubated in media containing EGTA, which are almost Ca2+-free. It is possible that the CaR is partially desensitized under physiological conditions and thus responds in vivo only to higher than normal concentrations of [Ca2+]i. If so, it is likely that secretogogue-induced vesicle acidification will be mediated both by the CaR and the entry of Ca2+ through L-type calcium channels, which was found to occur at concentrations of >1.0 mM. PF cells are depolarized by exposure to greater than resting concentrations of [Ca2+]i (21). It is conceivable that stimulation of the CaR contributes to the [Ca2+]i-induced depolarization of PF cells. Whether or not this is so, the depo-
lizarization is probably linked to the opening of L-type calcium channels, which are voltage-dependent, and thus to the activation of the parallel PKC-dependent pathway of vesicle acidification.

The development of a membrane potential difference limits the cytosol, where it is synthesized, into the vesicles in which it is stored is driven more by the proton gradient than by the potential difference across the vesicular membrane (18). Most of the vesicles of nonstimulated PF cells are not acidic because the development of a membrane potential difference limits acidification until the vesicular Cl⁻ channel opens in response to stimulation. It thus seems likely that the loading of 5-HT into vesicles is episodic and greatest after stimulus-induced vesicle acidification. The potential-driven uptake of 5-HT into the vesicles of resting cells is probably small in comparison with the pH gradient-driven uptake into the vesicles of secretogogue-stimulated cells. In fact, this enhancement of 5-HT uptake by secretogogue stimulation has actually been demonstrated (18). In contrast, the stability of 5-HT within vesicles may be decreased and its osmotic activity may be increased by various products within vesicles (25).

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