Ca²⁺-Evoked Serotonin Secretion by Parafollicular Cells: Roles in Signal Transduction of Phosphatidylinositol 3'-kinase, and the γ and ζ Isoforms of Protein Kinase C

Kuo-peing Liu,¹ Shu-chi Hsiung,¹ Mella Adlersberg,¹ Todd Sacktor,² Michael D. Gershon,³ and Hadassah Tamir^{1,3}

¹Division of Neuroscience, New York State Psychiatric Institute, New York, New York 10032, ²Department of Pharmacology, Physiology, and Neurology State University of New York Health Science Center, New York, New York 11203, and ³Department of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Parafollicular (PF) cells secrete 5-HT in response to stimulation of a G-protein-coupled Ca²⁺ receptor (CaR) by increased extracellular Ca²⁺ ($\uparrow$ [Ca²⁺]_e). We tested the hypothesis that protein kinase C (PKC) participates in stimulus–secretion coupling. Immunoblots from membrane and cytosolic fractions of isolated PF cells revealed conventional (α , β I, and γ), novel (δ and ϵ), and atypical (ι/λ and ζ) PKCs. Only PKC γ was found to have been translocated to the membrane fraction when secretion of 5-HT was evoked by $\uparrow$ [Ca²⁺]_e or phorbol esters. Although phorbol downregulation caused PKC γ to disappear, secretion was only partially inhibited. A similar reduction of $\uparrow$ [Ca²⁺]_e-evoked secretion was produced by inhibitors of conventional and/or novel PKCs (Gö 6976, calphostin C, and pseudoA), and these compounds did not inhibit secretion at all when applied to phorbol-downregulated cells. In contrast, the phorbol downregulation-resistant component of secretion was

abolished by pseudoZ, which inhibits the atypical PKC ζ . Stimulation of PF cells with $\uparrow$ [Ca²⁺]_e increased the activity of immunoprecipitated PKC ζ (but not PKC ι/λ), and the activity of this PKC ζ was inhibited by pseudoZ. PF cells were found to express regulatory (p85) and catalytic (p110 α and p110 β) subunits of phosphatidylinositol 3'-kinase (PI3'-kinase). $\uparrow$ [Ca²⁺]_e increased the activity of immunoprecipitated PI3'-kinase; moreover, PI3'-kinase inhibitors (wortmannin and LY294002) antagonized secretion. We suggest that PKC isoforms mediate secretion of 5-HT by PF cells in response to stimulation of the CaR. PKC involvement can be accounted for by PKC γ and an isoform sensitive to inhibition by pseudoZ, probably PKC ζ , which is activated via PI3'-kinase.

Key words: Ca²⁺ receptor; serotonin secretion; protein kinase C γ ; protein kinase C ζ ; phosphatidylinositol 3'-kinase; thyroid parafollicular cell

Serotonin (5-HT) and calcitonin are co-stored in the secretory vesicles of parafollicular (PF) cells (Zabel, 1984; Barasch et al., 1987b). As a result, PF cells secrete both transmitters when stimulated by their natural secretagogue, increased extracellular Ca²⁺ ($\uparrow$ [Ca²⁺]_e) (Nunez and Gershon, 1978). PF cells are able to respond to $\uparrow$ [Ca²⁺]_e because they express a plasmalemmal Ca²⁺ receptor (CaR) that is activated by changes in the extracellular Ca²⁺ concentration and polyamines, such as protamine (Herbert and Brown, 1995; Tamir et al., 1996). The CaR is G-protein-coupled (Herbert and Brown, 1995; Ruat et al., 1996) and has been demonstrated to be linked to Gi and Gq (Tamir et al., 1996). After the activation of the CaR by $\uparrow$ [Ca²⁺]_e, PF cells depolarize (as a result of an increase in a nonselective plasma membrane cation conductance) (McGehee et al., 1997), secretory vesicles acidify (Tamir et al., 1994b), and 5-HT is secreted (Tamir et al., 1990, 1994b; McGehee et al., 1997).

CaR stimulation leads to the activation of multiple signal transduction pathways. One of these pathways, which causes secretory vesicles to acidify, involves the coupling of the CaR via Gi

to phosphatidylinositol-specific phospholipase C (PI-PLC) (Herbert and Brown, 1995; Ruat et al., 1996; Tamir et al., 1996). The inositol trisphosphate (IP₃) generated by PI-PLC mobilizes Ca²⁺ from internal stores ([Ca²⁺]_i). The $\uparrow$ [Ca²⁺]_i activates a signaling cascade that involves, sequentially, calcium–calmodulin, nitric oxide synthase, guanylyl cyclase, and protein kinase G. This signal transduction pathway enables Cl[−] to pass through vesicular channels and act as a counterion for the transport of H⁺, so that the vesicles of secretagogue-stimulated cells become acidic. Although the activation of PI-PLC also leads to the formation of diacylglycerol and the consequent stimulation of isoforms of protein kinase C (PKC), PKC does not appear to contribute to the CaR-induced acidification of vesicles. Vesicle acidification and secretion can be uncoupled and thus are the result of different signal transduction mechanisms.

Stimulus–secretion coupling in PF cells is complex. PKC, however, appears to play an important role in the secretory process. Phorbol esters stimulate 5-HT secretion, and secretion in response to phorbol esters is abolished by phorbol ester downregulation of PKC (Tamir et al., 1990; McGehee et al., 1997). Nevertheless, despite the ability of phorbol ester downregulation of PKC to block phorbol ester-induced secretion, downregulation only slightly inhibits 5-HT secretion when it is evoked by $\uparrow$ [Ca²⁺]_e. This observation suggests that one or more phorbol ester-sensitive isoforms of PKC contribute to secretory signaling, but additional effectors, which are not recruited or downregulated

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Correspondence should be addressed to Dr. Hadassah Tamir, Division of Neuroscience, New York State Psychiatric Institute, 722 West 168th Street, New York, NY 10032. E-mail: ht3@columbia.edu.

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by phorbol esters, must also be involved in stimulus–secretion coupling from the CaR. These additional effectors could be atypical isoforms of PKC (aPKC), such as ι/λ and/or ζ because, in contrast to conventional (cPKC) and novel (nPKC) isoforms, aPKCs are neither activated nor downregulated by phorbol esters (Nishizuka, 1992; Newton, 1995). We therefore analyzed the role played by aPKCs in stimulus–secretion coupling after activation of the CaR. Data suggest that PKC γ and PKC ζ mediate PF cell 5-HT secretion in response to stimulation of the CaR and that phosphatidylinositol 3'-kinase (PI3'-kinase) participates in activating PKC ζ .

MATERIALS AND METHODS

Isolation of PF cells. Fresh sheep thyroid glands were obtained from a nearby kosher abattoir. The glands were dissociated with trypsin, and PF cells were isolated by “phagocytic chromatography” as previously described (Bernd et al., 1981; Barasch et al., 1987b, 1988; Cidon et al., 1991). This method uses thyrotropin (TSH) to activate the follicular cells, which become phagocytic in response to TSH. When a suspension of thyroid cells stimulated by TSH is passed through a Sepharose-thyroglobulin column, the follicular cells bind to the column, whereas the PF cells pass through in the void volume. Red blood cells are then removed from the suspension by centrifugation through a layer of Ficoll. Approximately 97% of the cells of the final preparation are parafollicular; the remainder are mainly fibroblasts, and there are no detectable follicular cells. Purified PF cells were cultured overnight at 37°C in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum and buffered by CO₂ to allow them to recover from the isolation procedure.

Analysis of 5-HT secretion from PF cells. Secretion by isolated PF cells (1×10^6 cells/ml) was induced by adding secretagogues, Ca²⁺ (5.0 mM, 10 min), protamine chloride (1.0 μ M, 20 min), or phorbol 12-myristate, 13-acetate (PMA; 300 nM, 30 min) to the medium at 37°C. Control cells were incubated in the presence of 1.0 mM EGTA. When the effect of inhibitors was studied, cells were preincubated with the indicated inhibitor for 30 min before the addition of secretagogue. The stimulus was terminated by quick chilling (on ice) and centrifuging the cells (800 $\times$ g). 5-HT and 5-hydroxyindole-acetic acid were extracted from the pelleted cells, and their supernatant was measured by reverse phase HPLC with an electrochemical detector (Tamir et al., 1994a). To compare results obtained in different preparations, the amounts of secreted 5-HT (in the supernatant) were normalized to the cellular 5-HT content (pellet). To estimate the secretagogue-induced increment in 5-HT release, the normalized 5-HT content of the supernatant of control cells was subtracted from that in the supernatant obtained from cells subjected to stimulation.

Downregulation of PKC by PMA. Isolated PF cells (10^7 cells) were incubated overnight in the presence of 1.0 μ M PMA (Nishizuka, 1992; Newton, 1995). The incubated cells were rinsed three times with PBS before exposure to another secretagogue. The extent of downregulation was tested by Western blot analysis or by the ability of the cells to secrete in response to PMA activation.

Gel electrophoresis and immunoblotting. Purified sheep PF cells ($\sim 10^7$) were harvested, washed with PBS, and homogenized in 300 μ l of 50 mM Tris buffer, pH 7.4, containing EDTA (1.0 mM), EGTA (2.0 mM), phenylmethanesulfonyl fluoride (1.0 mM), aprotinin (100 μ g/ml), and leupeptin (100 μ g/ml). The homogenate was centrifuged at 100,000 $\times$ g for 1 hr at 4°C to separate the membrane fraction (pellet) from cytosol. The pellet was solubilized in the above buffer containing Triton X-100 (1.0%). Proteins (100 μ g) were separated by 8.5% SDS-PAGE (Tamir et al., 1990). After separation, the proteins were electroblotted onto nitrocellulose membranes, and protein bands were visualized by staining the blots for 2 min in Ponceau solution (0.02% in 0.3% trichloroacetic acid). The blots were then washed and exposed to polyclonal antibodies raised against isozyme-specific PKC (1 μ g/ml, overnight at 4°C) followed by exposure to goat anti-rabbit secondary antibodies labeled with horseradish peroxidase (diluted 1:1000; Jackson ImmunoResearch, West Grove, PA). The membrane was then treated with chemiluminescent solution according to the manufacturer's directions (Amersham, Arlington Heights, IL) and exposed to film. Extracts of an HC11 mouse mammary epithelial cell line that overexpress either human PKC α or rat PKC β I were used as markers in identifying these isoforms of PKC. These cells were constructed by Dr. Eric Slosberg in Dr. B. Weinstein's laboratory (Columbia University Medical School) and kindly supplied to us. For

detection of subunits of PI3'-kinase the proteins from the total cell lysate (100 μ g) were solubilized, separated by SDS-PAGE, and blotted onto a nitrocellulose membrane. Blots were washed and exposed to polyclonal antibodies raised against p85 α , p110 α , and p110 β (1 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by chemiluminescence as described above.

Assay of aPKCs ζ and ι/λ . Purified PF cells (10^7) were harvested, washed with PBS, and resuspended in Ca²⁺-free Earl's buffered salt solution containing 1 mM MgCl₂. Cells were incubated in 5.0 mM Ca²⁺ (activated) for 10 min at 37°C. Control cells were incubated in the presence of 1.0 mM EGTA. When the effects of PI3'-kinase inhibitors were studied, cells were incubated with 100 μ M LY294002, 100 nM wortmannin, or 40 μ M pseudoZ for 30 min before the addition of Ca²⁺. PKC ζ and ι/λ activities were measured by using minor modifications of previously published methods (Gomez et al., 1997; Herrera-Velitz et al., 1997). After treatment, cells were washed in PBS and lysed for 1 hr at 4°C in 150 μ l of lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mM Na-vanadate, 100 mM okadaic acid, 100 μ g/ml aprotinin, 100 μ g/ml leupeptin, 0.5 mM phenylmethanesulfonyl fluoride, 12.5 μ g/ml pepstatin, and 1 mM benzamide). Lysates (750 μ g of protein) were microcentrifuged for 10 min at 13,000 rpm, and PKC ζ or PKC ι/λ was immunoprecipitated with rabbit polyclonal anti-PKC ζ (2 μ g/sample; Santa Cruz) or with mouse monoclonal anti-PKC ι/λ (1:100 dilution; Transduction laboratories, Lexington, KY) for 1–2 hr followed by the addition of 30 μ l of protein-A/G plus agarose beads (Santa Cruz) and incubated for an additional 1 hr at 4°C. The agarose–antigen–antibody complex was centrifuged and washed once with lysis buffer and twice with 10 mM Tris-HCl, pH 7.4. Beads were resuspended in 15 μ l of kinase buffer (50 mM Tris-HCl, pH 7.5, and 5 mM MgCl₂). Kinase activity was measured in the immunoprecipitates using myelin basic protein as a substrate (5 μ g, 21 kDa). In the indicated experiments, pseudoZ was added to the washed immunoprecipitate before assay of activity. The assay was initiated by the addition of 25 μ M ATP and 5 μ Ci of [γ -³²P]ATP (3000 Ci/mmol; Amersham) in 5 μ l of kinase buffer, continued for 30 min at room temperature, and stopped by boiling samples in Laemmli sample buffer. Samples were loaded onto a 12.5% polyacrylamide gel and subjected to electrophoresis. The gels were fixed, vacuum-dried, and exposed to Eastman Kodak (Rochester, NY) BioMax imaging film. Kinase activity was quantified by densitometric analysis of the films (Micro Computer Imaging Device; Imaging Research Inc., St. Catharines, Ontario, Canada).

Assay of PI3'-kinase activity. PF cells were washed, activated, and lysed as described above. PI3'-kinase activity was measured by using minor modifications of previously published methods (Ettinger et al., 1996; Herrera-Velitz and Reiner, 1996). Aliquots of cell lysates (750 μ g of protein) were immunoprecipitated with polyclonal antibodies to the p85 subunit of PI3'-kinase (5 μ g/sample; Santa Cruz) and allowed to incubate on ice for 60 min. Protein A/G-agarose (30 μ l) was then added, and incubation was continued for 60 min at 4°C. After centrifugation of the agarose–antigen–antibody complex, the pellets were washed with lysis and Tris buffers as described above. The complex was suspended in sonicated 20 μ g soybean phosphatidylinositol and 35 μ l of kinase assay buffer (30 mM HEPES, 30 mM MgCl₂, and 200 μ M adenosine). The reaction was initiated by the addition of 50 μ M ATP and 10 μ Ci of [γ -³²P]ATP in 5 μ l of kinase buffer; incubation was performed for 10 min, and the reaction was stopped by the addition of 100 μ l of 1N HCl. Lipids were extracted with 200 μ l of chloroform:methanol (1:1), spotted onto silica gel TLC plates, and developed in a mobile phase consisting of chloroform, methanol, water, and NH₄OH (18:14:3:1 v/v/v/v). Spots corresponding to phosphatidylinositol 3-phosphate were detected by autoradiography and identified on the basis of their co-migration with a known standard. Kinase activity was quantified by cutting the spots corresponding to PI3-P from the plate and analyzing them by liquid scintillation counting.

Drugs and chemicals. All drugs and chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified. The PKC inhibitors, calphostin C, and chelerythrine were obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). Gö 6976, PKC inhibitor, and LY294002, an inhibitor of PI3'-kinase, were purchased from Biomol Research Labs (Plymouth Meeting, PA). Wortmannin was purchased from Alexis Corp. (San Diego, CA). Protein A/G plus agarose beads was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to the p85 α (z-8) subunit of PI3'-kinase and antibodies to the 110 β subunit forms of PI3'-kinase, with the corresponding peptide, were purchased from Santa Cruz. Polyclonal rabbit anti-PKC α , PKC β I, and PKC γ , with their respec-

Figure 1. Many isoforms of PKC are found in PF cell membrane and cytosolic fractions. Cytosol (Cyt) and membrane (Mem) fractions were obtained from nonstimulated PF cells. Proteins in the fractions (100 μ g) were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with monospecific antibodies to the indicated isoforms of PKC. Bound antibodies were visualized by chemiluminescence. PKC isoforms are indicated at the left, and the molecular weight is shown at the right. The standards (Std) used for comparison were derived from HC11 cells that overexpress PKC α and PKC β I or from a commercial source. For PKC α , PKC β I, and PKC γ (Peptide, top right), the specificity of the antibodies used for identification of PKC isoforms was verified by blocking immunostaining with the corresponding peptide antigen at 10 μ g/ml.

tive peptides, and FITC-conjugated goat anti-rabbit IgG, anti-mouse IgG, and Earl's balanced salt solution were purchased from Life Technologies (Grand Island, NY.). Monoclonal antibodies to PKC η , μ , θ , and λ were purchased from Transduction Laboratories. Polyclonal antibodies to PKC β I, δ , ϵ , and ζ were prepared as previously described (Sacktor et al., 1993). Goat anti-rabbit IgG coupled to rabbit horseradish peroxidase was purchased from Kirkegaard and Perry (Gaithersburg, MD). Antibodies to the 110 kDa subunit of PI3'-kinase were contributed by Dr. Jonathan M. Backer (Albert Einstein College of Medicine) (Rordorf-Nikolic et al., 1995). Myristoylated protein kinase C $_{19-27}$, cell-permeable cPKC inhibitors (pseudoA), and the cell-permeable myristoylated PKC ζ pseudosubstrate, myr-SIYRRGARRWRKL (pseudoZ) were purchased from Quality Controlled Biochemicals, Inc. (Hopkinton, MA). Silica gel G 60, was purchased from EM Science (Gibbstown, NJ). Solvents used with all compounds were routinely tested for their effects on secretion. None was found at the concentrations used.

RESULTS

Several isoforms of PKC are expressed in PF cells

To determine which PKC isoforms are expressed in PF cells, cell extracts were analyzed by immunoblotting with isozyme-specific antibodies. Membrane and soluble fractions of purified PF cell homogenates were analyzed separately. Where possible, the specificity of the immunoreactivity of PKCs was verified by absorption of antibodies with isozyme-specific peptides. Every known PKC isoform except β II and θ was detected in PF cells (Fig. 1). All were, in resting cells, more concentrated in cytosolic than membrane fractions; nevertheless, a substantial amount of ζ and a small amount of γ immunoreactivity was found in membranes, even those isolated from nonstimulated PF cells. It should be noted that all the antibodies used for immunoblotting were tested for specificity. In no instance was cross-reactivity observed.

To stimulate secretion, isolated PF cells were exposed to PMA (0.3 μ M) or $\uparrow$ [Ca $^{2+}$] $_e$ (5.0 mM) for 10–30 min (the time course of 5-HT secretion induced by the two secretagogues). Cytosolic and membrane fractions were then obtained. Immunoblots were used to determine whether stimulation had induced a translocation of specific isoforms of PKC to membranes. PF cells secreted 5-HT after exposure to PMA or $\uparrow$ [Ca $^{2+}$] $_e$ (Tamir et al., 1996; McGe-

Figure 2. Phorbol ester (PMA) activates and downregulates PKC γ . Isoforms of PKC were investigated by Western blot analysis as in Figure 1. *A*, PF cells were treated with PMA (300 nM) for 0, 0.5, or 6.0 hr before collection and fractionation. The relative amount of PKC γ decreased in the cytosol and increased in the membrane fraction as a function of time after exposure to PMA. *B*, PF cells were treated with PMA (1.0 μ M) for 0 or 24 hr before collection and fractionation. After the prolonged exposure to PMA, PKC γ could no longer be detected in either the cytosol or membrane fractions.

hee et al., 1997), but no translocation of PKC to membranes could be detected for any isozyme except for PKC γ (Fig. 2). Similar data were obtained by immunocytochemically examining cells in conjunction with laser-scanning confocal microscopy (data not illustrated), which, like immunoblotting, failed to demonstrate the membrane translocation of isoforms of PKC other than PKC γ . After PF cells were exposed for 24 hr to PMA, only PKC γ disappeared from both membrane and cytosolic fractions (Fig. 2). PKC γ thus appears to be activated immediately after exposure to a phorbol ester and is eventually downregulated. It is surprising that phorbol esters do not cause a detectable translocation or downregulation of PKCs (novel and Ca $^{2+}$ -dependent) other than PKC γ .

The ability of phorbol esters to activate cPKCs and nPKCs is related to their binding to the regulatory C1 domains of these classes of PKC isozymes (Newton, 1995, 1997). In contrast to the cPKCs and nPKCs, phorbol esters do not bind to the C1 domain of the aPKCs; therefore, PMA would not have been expected to be able to stimulate α , λ , or ζ . Conceivably, translocation of aPKC isoforms in Western blots might be masked by the high levels associated with membrane fractions derived from nonstimulated PF cells. Activated PKC isozymes bind to anchoring proteins termed RACKs (Mochly-Rosen, 1995), which are believed to be positioned in close proximity to the isozyme substrate. These PKCs, therefore, may be closely associated with RACKs in or near the plasma membrane even in nonstimulated PF cells. For this reason, it may not be possible to demonstrate translocation of PKC isoforms even when they are activated.

Downregulation of PKC by chronic exposure to PMA partially inhibits $\uparrow$ [Ca $^{2+}$] $_e$ -induced 5-HT secretion

To determine whether cPKC or nPKC isozymes participate in mediating the secretory response to $\uparrow$ [Ca $^{2+}$] $_e$, the effects of down-regulating these isozymes by chronically exposing isolated PF cells to PMA were investigated. Conditions were the same as those that were found to fully down-regulate PKC γ . The extent of downregulation was tested by measuring secretion in response to stimulation with PMA. Before treatment with PMA, exposure of PF cells to $\uparrow$ [Ca $^{2+}$] $_e$ (5.0 mM) produced an ~2.5-fold increase in the release of 5-HT (Fig. 3). After incubation overnight with

Figure 3. Effect of inhibitors of the regulatory and catalytic domains of PKC on Ca^{2+} - and PMA-induced secretion. The natural PF secretagogue $\uparrow[\text{Ca}^{2+}]_e$ (5 mM, 10 min) and PMA (300 nM, 30 min) were used to evoke 5-HT secretion. Data are presented as a percentage of 5-HT released induced by either Ca^{2+} or PMA of resting release (assigned control; mean $\pm$ SE). The numbers of determinations are indicated in each bar. The effects of PKC inhibitors on the secretion elicited by each secretagogue were then assessed. In addition, PKC was downregulated by overnight exposure to PMA (1.0 μM). The regulatory site inhibitors calphostin C (5 μM) and Gö 6976 (0.5 μM), as well as PKC downregulation, all partially and weakly antagonized the secretion of 5-HT evoked by $\uparrow[\text{Ca}^{2+}]_e$. In contrast, the catalytic inhibitor chelerythrine (5.0 μM) strongly inhibited secretion of 5-HT evoked by $\uparrow[\text{Ca}^{2+}]_e$ from both downregulated and nondownregulated cells ($85 \pm 6\%$; $p < 0.001$). All of the inhibitors (calphostin C, chelerythrine, and Gö 6976) and phorbol ester downregulation fully inhibited the secretion evoked by PMA ($90 \pm 5\%$ inhibition; $p < 0.001$).

PMA (1.0 μM), the $\uparrow[\text{Ca}^{2+}]_e$ -induced release of 5-HT declined by $\sim 35\%$ (Fig. 3). A specific cPKC inhibitor, Gö 6976 (0.1–1.0 μM) (Martiny-Baron et al., 1993), and calphostin C (5 μM) (Kobayashi and Nakano, 1989; Rotenberg et al., 1995), which at low concentrations inhibits both cPKCs and nPKCs, antagonized the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT to approximately the same extent as downregulation of PKC. Neither Gö 6976 nor calphostin C antagonizes aPKC isoforms. In contrast to their relatively small effect on $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT, downregulation of PKC γ , calphostin C, and Gö 6976 almost completely abolished the ability of PF cells to secrete in response to PMA stimulation (Fig. 3B). In contrast to calphostin C and Gö 6976, which only partially antagonized $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion, chelerythrine almost completely blocked the secretion of 5-HT induced by $\uparrow[\text{Ca}^{2+}]_e$. Chelerythrine, moreover, was equally effective regardless of whether PKC had previously been downregulated by exposure to a phorbol ester (Fig. 3A). Because chelerythrine acts on the PKC catalytic domain (Herbert et al., 1990), the action of chelerythrine is independent of the presence or absence of a phorbol ester-binding regulatory region. Chelerythrine would thus be expected to inhibit aPKCs as well as conventional and novel isoforms. It should be noted that the secretagogues (PMA and $\uparrow[\text{Ca}^{2+}]_e$) were used at concentrations that were within the linear range of the secretory response. The observations that calphostin C and Gö 6976 partially inhibit the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT suggest that one or more cPKCs or nPKCs participate in the mediation of the response. These data are consistent with the observation that the cPKC PKC γ is translo-

cated to the plasma membrane when cells are stimulated by $\uparrow[\text{Ca}^{2+}]_e$. The fact that calphostin C, which inhibits both nPKCs and cPKCs, is not more effective than Gö 6976, which inhibits only cPKCs, suggests that nPKCs contribute relatively little to the response. In any case, the sum of the effects of cPKCs and nPKCs does not account for the major component of $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion. PKC isoforms in these categories, however, probably do account for almost all of the secretion of 5-HT evoked by a phorbol ester. Because the ability of chelerythrine to almost completely abolish $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion is consistent with the involvement of an aPKC, these isoforms were investigated further.

Cell-permeable pseudosubstrates of PKC inhibit 5-HT secretion

Membrane-permeable pseudosubstrate peptides, pseudoA and pseudoZ, as well as isozyme-specific PKC inhibitors were used to evaluate the role played by aPKCs in the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT. These pseudosubstrate peptides suppress PKC activity by interacting with the substrate-binding site in the catalytic domain of the enzyme and thus are relatively specific inhibitors of the PKC isoforms with which they interact (Shen and Buck, 1990; Huntley et al., 1997). PseudoA has a sequence conserved in the pseudosubstrate region of PKC isotypes α , β , and γ . PseudoZ has a sequence identical to that of the pseudosubstrate region of PKC ζ , which differs significantly from those found in PKC α , β , or γ . PseudoZ is known to inhibit PKC ζ (Zhou et al., 1997; Sajan et al., 1999) but has not previously been shown not to inhibit any other isoforms of PKC (Standaert et al., 1997). We found that pseudoA (40 μM , 30 min exposure) inhibits the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT. The degree of inhibition of 5-HT secretion by pseudoA was comparable with that produced by the phorbol ester downregulation of PKC (Fig. 4). These data are compatible with the idea that the effect of PMA is mediated by a cPKC and that a cPKC also makes a partial contribution to the mediation of $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT. In the absence of downregulation of PKC by prolonged exposure to PMA, pseudoZ (40 μM , 30 min exposure) inhibited the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT ($65 \pm 5\%$; Fig. 4). Increasing the concentration of pseudoZ to 80 μM did not affect the degree of inhibition of 5-HT secretion. In contrast, when PKC γ was downregulated by PMA, pseudoZ inhibited the residual secretion by $90 \pm 5\%$. When both pseudoA and pseudoZ were added to down-regulated PF cells, the effect of the combination was essentially the same as that of pseudoZ alone. The addition of both pseudoA and pseudoZ to cells that had not been down-regulated by chronic exposure to PMA produced approximately the same degree of inhibition of $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT ($92 \pm 5\%$) as the combination of down regulation plus pseudoZ (Fig. 4). These data indicate that isoforms of PKC mediate stimulus–secretion coupling in PF cells. The role of PKC can be accounted for by the involvement of a combination of a cPKC (PKC γ) and an isoform of PKC that is inhibited by pseudoZ.

PI3'-kinase participates in signal transduction from the CaR

Recent studies have demonstrated that both nPKCs and aPKCs are activated by products of PI3'-kinase (Nakanishi et al., 1993; Toker et al., 1994; Derman et al., 1997; Herrera-Velitz et al., 1997; Standaert et al., 1997; Chou et al., 1998). It is thus possible that PI3'-kinase is responsible for activating the pseudoZ-sensitive

Figure 4. Secretion evoked by receptor stimulation is dependent on phorbol ester-sensitive and -insensitive forms of PKC. The effects of $\uparrow[\text{Ca}^{2+}]_e$ (5 mM) on 5-HT-induced secretion were assayed in the presence and absence of PKC inhibitors. Downregulation of $\text{cPKC}\gamma$ was achieved by exposing cells overnight to 1 μM PMA. PseudoA and pseudoZ were added 30 min before the addition of the secretagogue. The resting release of 5-HT was taken as a control and the data for the evoked release of 5-HT are presented as a percent of control, which was assigned a value of 100% (mean $\pm$ SE). In the absence of phorbol ester-downregulation of PKC, the $\uparrow[\text{Ca}^{2+}]_e$ -evoked released of 5-HT was inhibited by both pseudoA ($35 \pm 4\%$; $p < 0.005$) and pseudoZ ($65 \pm 6\%$; $p < 0.005$). After the phorbol ester downregulation of PKC, the residual secretion of 5-HT could no longer be inhibited by pseudoA but now was blocked by pseudoZ ($90 \pm 5\%$; $p < 0.001$). The combination of pseudoA and pseudoZ abolished secretion regardless of whether cells had previously been phorbol ester-downregulated. These data are consistent with the idea that an aPKC , such as $\text{PKC}\zeta$, contributes to stimulus–secretion coupling and is responsible for mediating the downregulation-resistant component of the $\uparrow[\text{Ca}^{2+}]_e$ -evoked release of 5-HT.

isoform of PKC (such as $\text{PKC}\zeta$) after stimulation of the CaR. The role of $\text{PI}3'$ -kinase in stimulus–secretion coupling was thus investigated. $\text{PI}3'$ -kinase is a heterodimer composed of an 85 kDa regulatory subunit ($\text{p}85\alpha$) and a 110 kDa catalytic subunit ($\text{p}110$) (Carpenter et al., 1990; Shibasaki et al., 1991; Kapeller and Cantley, 1994). Several isoforms of this enzyme have been detected (α , β , and γ) (Thomason et al., 1994; Rordorf-Nikolic et al., 1995; Ptasnik et al., 1996; Kurosu et al., 1997; Tang and Downes, 1997). Immunoblot analysis demonstrated that subunits of $\text{PI}3'$ -kinase, $\text{p}85$ and $\text{p}110$ ($\alpha + \beta$), are expressed in PF cells (Fig. 5). The antibodies to $\text{p}110\beta$ were specific because their immunoreactivity was abolished after absorption with the corresponding peptide antigen. Results obtained with Western blots were confirmed by immunocytochemical studies that demonstrated the presence of $\text{p}110\alpha + \beta$ as well as $\text{p}85$ in PF cells (data not shown). We therefore investigated the effects of secretory stimuli on the activity of $\text{PI}3'$ -kinase.

Stimulation of secretion by exposing PF cells to $\uparrow[\text{Ca}^{2+}]_e$ did not alter the apparent intracellular distribution of $\text{p}85$ immunoreactivity as assessed by immunocytochemistry. Stimulation of PF cells by $\uparrow[\text{Ca}^{2+}]_e$ did, however, increase $\text{PI}3'$ -kinase activity by $50 \pm 5\%$ (Fig. 6A,B). Wortmannin (100 nM) reduced the basal level of $\text{PI}3'$ -kinase activity and abolished the increment induced by exposure to $\uparrow[\text{Ca}^{2+}]_e$ (Fig. 6A,B). Wortmannin (≤ 100 nM) specifically binds to and inhibits $\text{p}110$ (the catalytic subunit of

Figure 5. Isoforms of $\text{PI}3'$ -kinase are present in PF cells. Proteins from the total lysate of isolated PF cells (100 μg) were solubilized and separated by SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with monospecific antibodies directed against subunits of $\text{PI}3'$ -kinase. Bound antibodies were visualized by chemiluminescence. Lane 1, $\text{p}85$; lane 2, $\text{p}110\alpha$; lane 3, $\text{p}110\beta$; lane 4, control preparation showing that $\text{p}110\beta$ immunoreactivity is removed by absorption with the corresponding peptide antigen at a ratio of 2:1.

$\text{PI}3'$ -kinase) (Yano et al., 1993; Okada et al., 1994). The $\uparrow[\text{Ca}^{2+}]_e$ -induced increase in $\text{PI}3'$ -kinase activity was also prevented by LY294002 (100 μM ; data not illustrated), a compound that is not structurally related to wortmannin but that also inhibits $\text{PI}3'$ -kinase and does not antagonize other protein kinases that may be inhibited by wortmannin (Vlahos et al., 1994).

The effects of inhibitors of $\text{PI}3'$ -kinase on $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT were determined. Wortmannin (10–100 nM) was applied to cells in which $\text{PKC}\gamma$ had previously been downregulated by chronic exposure to PMA, so that the contribution of $\text{PI}3'$ -kinase to the activation of phorbol ester-independent transduction mechanisms could be examined in isolation. Wortmannin effectively antagonized the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT (Fig. 7). Results obtained with wortmannin were confirmed by administering LY294002 (20–100 μM). The data obtained with LY294002 were not distinguishable from those obtained with wortmannin. Both compounds were equally able to inhibit the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT by PF cells ($60 \pm 5\%$; Fig. 7). These data support the idea that $\text{PI}3'$ -kinase participates in stimulation–secretion coupling after activation of the CaR. The residual secretion of 5-HT from PF cells that remained after the addition of wortmannin or LY294002 after the downregulation of $\text{PKC}\gamma$ (Fig. 7) may be explained by the existence of a parallel transduction pathway that is independent of $\text{PI}3'$ -kinase.

Because it is conceivable that Ca^{2+} elicits effects that are not mediated by the CaR, additional experiments were performed with protamine as a CaR agonist. Like $\uparrow[\text{Ca}^{2+}]_e$, protamine evoked 5-HT secretion (to $135 \pm 8\%$ of control, a value not significantly different from that evoked by $\uparrow[\text{Ca}^{2+}]_e$, $133 \pm 10\%$ of control). Wortmannin (100 nM; $p < 0.001$ vs control) and LY294002 (100 μM ; $p < 0.001$ vs control) blocked the protamine-induced secretion.

$\text{PKC}\zeta$ participates in signal transduction from the CaR

The activity of $\text{PKC}\zeta$ was assayed to test the hypothesis that $\text{PKC}\zeta$ is activated by $\text{PI}3'$ -kinase and participates in secretion. $\text{PKC}\zeta$ was immunoprecipitated from PF cells that were or were not stimulated by $\uparrow[\text{Ca}^{2+}]_e$. The ability of the resulting immune complex to phosphorylate myelin basic protein was then measured. PseudoZ abolished enzyme activity, confirming that the measured activity was that of $\text{PKC}\zeta$ and suggesting that the inhibitor acts on the catalytic site of the enzyme (Fig. 8A,B). The activity of $\text{PKC}\zeta$ was significantly greater in $\uparrow[\text{Ca}^{2+}]_e$ -stimulated than in control cells (Fig. 8; $p < 0.001$; $n = 6$). The effect of exposure to $\uparrow[\text{Ca}^{2+}]_e$ on $\text{PKC}\zeta$ activity was abolished by wortmannin (Fig. 8A,B; 100 nM; $p < 0.001$; $n = 6$), confirming that the response was $\text{PI}3'$ -kinase-dependent. Chelerythrine inhibited

Figure 6. PI3'-kinase activity in PF cells is increased by $\uparrow[\text{Ca}^{2+}]_e$ and is antagonized by PI3'-kinase inhibitors. PF cells were incubated for 30 min in the absence or presence of wortmannin (100 nM). $\uparrow[\text{Ca}^{2+}]_e$ (5.0 mM) was used to stimulate the CaR in both sets of cells. Extracts were prepared, and equivalent amounts of protein (750 μg) were used for immunoprecipitation with antibodies to the p85 subunit of PI3'-kinase. The kinase activity of the immune complex was measured using phosphatidylinositol as a substrate. Lipids were extracted and analyzed by TLC and radioautography (*A*). Spots corresponding to phosphatidylinositol 3-phosphate (PI3-P) were cut out and analyzed by liquid scintillation (*B*). Radioactivity observed at the origin reflects residual, water-soluble ^{32}P -labeled material in the samples, the amount of which is not relevant to the results. PI3'-kinase activity is expressed as a percentage of that found in control (Cont) cells (average of 5 independent experiments). The elevation of PI3'-kinase induced by $\uparrow[\text{Ca}^{2+}]_e$ is significant ($50 \pm 5\%$; $p < 0.001$ vs the null hypothesis of 100%), as is its inhibition by wortmannin ($p < 0.001$, $\uparrow[\text{Ca}^{2+}]_e$ vs $\uparrow[\text{Ca}^{2+}]_e$ + wortmannin).

PKC ζ by $75 \pm 8\%$. These observations support the idea that stimulation of the CaR leads to the activation of PI3'-kinase, which in turn activates an aPKC. The inability of wortmannin to inhibit the resting activity of PKC ζ suggests that the basal activity of this enzyme is not the result of stimulation by PI3'-kinase. To determine whether PKC ϵ/λ , the other aPKC present in PF cells, also participates in the transduction of secretion in response to $\uparrow[\text{Ca}^{2+}]_e$, we assayed the activity of PKC ϵ/λ (Fig. 8C). The basal activity of PKC ϵ/λ , unlike that of PKC ζ , was not affected by stimulating cells with $\uparrow[\text{Ca}^{2+}]_e$ (Fig. 8C). These results strongly suggest that PKC ζ and not PKC ϵ/λ is activated by PI3'-kinase.

DISCUSSION

The current study was undertaken to identify the role(s) played by PKC in CaR-related stimulus–secretion coupling in PF cells. The physiological induction of secretion of 5-HT by the natural secretagogue $\uparrow[\text{Ca}^{2+}]_e$ was compared with the induction of secretion

Figure 7. $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT by PF cells is antagonized by PI3'-kinase inhibitors. PF cells were chronically treated with PMA to downregulate cPKC γ . Cells were pretreated for 30 min with wortmannin (Wort; 100 nM) or LY294002 (LY; 100 μM). Secretion was measured after the addition of $\uparrow[\text{Ca}^{2+}]_e$ (5.0 mM). Data are presented as a percentage of control (no secretagogue; mean $\pm$ SE). Both wortmannin and LY294002 inhibited the $\uparrow[\text{Ca}^{2+}]_e$ -induced secretion of 5-HT by PF cells. Note that the magnitude of inhibition by 100 nM wortmannin is approximately equal to that caused by 100 μM LY294002. Wortmannin is therefore more potent than LY294002, but both are effective inhibitors of the secretion of 5-HT. The inhibition of 5-HT induced by $\uparrow[\text{Ca}^{2+}]_e$ is significant ($60 \pm 5\%$; $p < 0.001$ vs the null hypothesis of 100%), as is its inhibition by LY294002 ($p < 0.001$, $\uparrow[\text{Ca}^{2+}]_e$ vs $\uparrow[\text{Ca}^{2+}]_e$ + LY294002).

by a phorbol ester. Eight isoforms of PKC, including each of the three main classes of PKC, conventional, novel, and atypical, were found to be expressed. The expression in PF cells of PKC γ , which was previously thought to be a brain-specific isoform (Nishizuka, 1995), may reflect the neural crest origin of PF cells and their relationship to neurons (Barasch et al., 1987a; Russo et al., 1992; Clark et al., 1995). The expression of many isoforms of PKC in a single cell is another characteristic that is reminiscent of neurons (Sacktor et al., 1993).

The CaR is known to occur in the CNS as well as in the endocrine cells of the thyroid and parathyroid. For example, the CaR has been detected and studied electrophysiologically in neurons of the rat subfornical nucleus (Washburn and Ferguson, 1998). The CaR is widespread in the brain and develops early in ontogeny (Chattopadhyay et al., 1997). The distribution of mRNA encoding the CaR in the brain (Rogers et al., 1997) and the gut (Cheng et al., 1999) is quite extensive. Stimulation of the brain CaR expressed in a transfected cell line activates phospholipase C and causes accumulation of IP $_3$ (Ruat et al., 1996). Phospholipase A $_2$ is also activated in these cells (Brown et al., 1993). It has been suggested that the CaR may enable neurons to respond to changes in the extracellular concentration of Ca^{2+} and, by activating Ca^{2+} -dependent Cl^- channels, to participate in regulating their Cl^- equilibrium potential (Brown et al., 1993). The lipid products of PI3'-kinase contribute to regulation of Cl^- transport (Feranchak et al., 1999), and we have found that the CaR activates PI3'-kinase.

Figure 8. PKC ζ but not PKC α/λ activity is increased in PF cells stimulated by $\uparrow[Ca^{2+}]_e$ and is antagonized by PI3'-kinase inhibitors or pseudoZ. PF cells were incubated for 30 min in the absence or presence of wortmannin (100 nM) or pseudoZ (40 μ M). $\uparrow[Ca^{2+}]_e$ (5.0 mM) was used to stimulate the CaR in both sets of cells. Extracts were prepared, and equivalent amounts of protein (750 μ g) were used for immunoprecipitation with antibodies to PKC ζ . PKC activity of the immune complex was measured using myelin basic protein (MBP) as a substrate. Proteins were separated by electrophoresis, dried gels were exposed to film (A), and the films were developed and quantified densitometrically (B). Note in the Western blots (A) that an equal amount of protein was immunoprecipitated for assay of PKC ζ under each of the experimental conditions studied. In contrast, the activity of PKC ζ was inhibited by wortmannin and pseudoZ. PKC ζ activity is expressed as a percentage of that found in control cells. The elevation of PKC ζ induced by $\uparrow[Ca^{2+}]_e$ is significant (40%; $p < 0.001$ vs the null hypothesis of 100%), as is its inhibition by wortmannin ($p < 0.001$, $\uparrow[Ca^{2+}]_e$ vs $\uparrow[Ca^{2+}]_e$ + wortmannin). PseudoZ completely abolished the PKC ζ activity. To investigate the possible activation of PKC α/λ by $\uparrow[Ca^{2+}]_e$, cells were activated as above, but extracted protein was immunoprecipitated (IP) with antibodies to PKC α/λ instead of PKC ζ (C). Note that the basal activity of PKC α/λ was unchanged by exposing cells to $\uparrow[Ca^{2+}]_e$ (87.8–89.8% of control).

Because phorbol ester downregulation of PKC reduced $\uparrow[Ca^{2+}]_e$ -induced secretion of 5-HT, it is apparent that at least one conventional and/or novel isoform of PKC participates in secretion. On the other hand, the observation that the effect of

phorbol ester downregulation is relatively minor suggests that secretion is not mediated entirely by isoforms of PKC that are sensitive to phorbol esters. Indeed, we detected the translocation to membrane of only a small amount of PKC γ . The ability of downregulation to abolish secretion induced by PMA demonstrates that the failure of this treatment to abolish $\uparrow[Ca^{2+}]_e$ -induced secretion cannot be explained by the incomplete downregulation of the activity of whatever conventional and/or novel isoforms of PKC are stimulated by phorbol esters. Downregulation, moreover, causes PKC γ to disappear. The secretagogue activity of PMA, therefore, can be entirely accounted for by activation of PKC γ (also see below). Atypical isoforms of PKC, which lack phorbol ester binding sites, are neither activated nor down-regulated by phorbol esters. We thus tested the hypothesis that an aPKC mediates the phorbol ester-independent component of the secretion of 5-HT induced by $\uparrow[Ca^{2+}]_e$.

PF secretion was evoked by $\uparrow[Ca^{2+}]_e$ and monitored after exposure to PMA to eliminate the contribution of PKC γ . Gö 6976, an inhibitor of conventional PKCs (Martiny-Baron et al., 1993), and calphostin C, an inhibitor of conventional and novel PKCs (Kobayashi and Nakano, 1989; Rotenberg et al., 1995), were unable to inhibit $\uparrow[Ca^{2+}]_e$ -induced secretion under these conditions. The resistance of nPKCs to inhibition by calphostin C may mirror their resistance to phorbol ester translocation, because calphostin C and phorbol esters bind to the same site on the enzymes. In contrast to Gö 6976 and calphostin C, the catalytically active PKC antagonist chelerythrine effectively blocked the secretion of 5-HT evoked by $\uparrow[Ca^{2+}]_e$. Secretion evoked by $\uparrow[Ca^{2+}]_e$ after the downregulation of PKC was also prevented by pseudoZ, an inhibitor of the aPKC, PKC ζ , but not by pseudoA, an inhibitor of cPKCs. PseudoA, however, did inhibit the $\uparrow[Ca^{2+}]_e$ -induced secretion of 5-HT in the absence of downregulation and did so to the same degree as calphostin C and Gö 6976; moreover, the combination of pseudoA and pseudoZ was fully able to prevent secretion. These observations are compatible with the idea that the CaR-induced secretion of 5-HT by PF cells involves the participation of both PKC γ and PKC ζ . The action of PKC γ is probably relatively minor in comparison with that of PKC ζ , because the secretion of 5-HT induced by $\uparrow[Ca^{2+}]_e$ after the downregulation of conventional PKC γ is strong. Although the participation of an nPKC in secretion has not been definitively ruled out, this involvement seems less likely than that of an aPKC for the following reasons: (1) The membrane translocation of an nPKC could not be demonstrated. This point is not, by itself, compelling, because PKC isoforms may be close to the membrane bound to RACKs even under resting conditions (Mochly-Rosen, 1995). (2) Calphostin C, which inhibits both cPKCs and nPKCs, did not antagonize $\uparrow[Ca^{2+}]_e$ -induced secretion more than did the cPKC inhibitor, Gö 6976. (3) After downregulation, calphostin C failed to inhibit $\uparrow[Ca^{2+}]_e$ -induced secretion at all (showing that the isoforms of PKC that mediate $\uparrow[Ca^{2+}]_e$ -induced secretion after PKC downregulation are calphostin C-resistant, which is not true of any nPKC). Direct studies, perhaps performed out with dominant negative mutants, will be necessary in the future to determine whether nPKCs are involved at all in PF cell secretion.

The idea that PKC ζ mediates the secretion of 5-HT induced by the CaR was tested further by measuring the activity of aPKCs in control and secretagogue-stimulated PF cells. Exposure to $\uparrow[Ca^{2+}]_e$ significantly increased PKC ζ activity but did not affect that of PKC α/λ ; moreover, pseudoZ, in a concentration that blocked secretion, also inhibited the activity of PKC ζ without

antagonizing that of PKC ϵ /λ when added to the cells before activation of the cells. It can thus be concluded that PKCζ is an important contributor to the secretion induced by stimulation of the CaR in PF cells. This is the first demonstration that an aPKC isoform is involved in stimulus–secretion coupling.

The mechanism by which aPKCs are activated is not yet totally clear; nevertheless, several studies have demonstrated that PKCζ can be stimulated by phosphatidylinositol polyphosphates, which are products of PI3'-kinase activity (Nakanishi et al., 1993; Toker et al., 1994; Derman et al., 1997). In the current study, PF cells were demonstrated to contain both the regulatory (p85) and catalytic subunits (p110, both α and β forms) of PI3'-kinase. Stimulation of PF cells with ↑[Ca²⁺]_e increased the activity of PI3'-kinase. This effect was abolished by two structurally unrelated inhibitors of PI3'-kinase, wortmannin and LY294002. Because these compounds also inhibited ↑[Ca²⁺]_e-induced secretion, PI3'-kinase activity is probably involved in stimulus–secretion coupling and may be the activator of PKCζ. Indeed, wortmannin fully inhibited the activity of PKCζ. The regulation of PKCζ by PI3'-kinase may occur via the phosphoinositide-dependent protein kinase-1 (PDK-1), which binds with high affinity to the PI3'-kinase lipid product phosphatidylinositol-3,4,5-trisphosphate. Previous studies have identified PDK-1 as the kinase that phosphorylates activation loop sites and activates PKCζ (Chou et al., 1998; Le Good et al., 1998).

The signal that couples an activated CaR to the stimulation of PI3'-kinase is not yet known. At least three modes of activation of PI3'-kinase have been reported. One is dependent on the stimulation of membrane receptor tyrosine kinases (Rordorf-Nikolic et al., 1995; Ptasznik et al., 1996). These proteins initiate the phosphorylation of specific tyrosine residues, located either in the receptors themselves or in adapter molecules. These phosphorylated proteins bind to the Src homology 2 domains of p85, which in turn regulates the activity of p110 (α, β, or δ). A second mode of PI3'-kinase activation is via the βγ subunits of G proteins, which activate the p110γ monomer (Tang and Downes, 1997). A third mechanism of PI3'-kinase activation involves βγ subunits working synergistically with a phosphotyrosyl peptide to activate the heterodimer enzyme p110β/p85 (Thomason et al., 1994; Kurosu et al., 1997). It is clear, therefore, that there are multiple species of PI3'-kinase that can be stimulated by the βγ components of G proteins. PF cells were found to contain the p110β and the p85 isoforms of PI3'-kinase. Both p110β and p85 were immunoprecipitated with antibodies to p85. Furthermore, the co-immunoprecipitate obtained from activated cells exhibited an elevated level of PI3'-kinase activity. The CaR has been demonstrated to be coupled to G proteins (Tamir et al., 1996); therefore, it is plausible that liberated βγ subunits stimulate PI3'-kinase in PF cells after activation of the CaR.

Previous observations have demonstrated that stimulation of the CaR leads to the activation of Gi and Gq (Tamir et al., 1996). As has previously been demonstrated (Brown et al., 1993; Emanuel et al., 1996; Tamir et al., 1996), CaR activation is coupled to a pertussis toxin-sensitive Gi-protein. Signaling via this pathway activates PI-PLC with the consequent production of DAG from phosphatidyl inositol. This is the transduction mechanism that is responsible for stimulus–acidification coupling (Tamir et al., 1996). Although the coupling of receptors to PI-PLC is more commonly via Gαq, Gαi also couples many receptors to PI-PLC. These include the m2 muscarinic receptor, thrombin, metabotropic glutamate, α₂-adrenergic, and others (Ashkenazi et al., 1989; Cotecchia et al., 1990; Masayuki et al., 1991; Dell'Ackqua et

al., 1993). The DAG released secondary to the activation of these G proteins probably stimulates the PKCγ that participates in stimulus–secretion coupling. By a parallel pathway, the βγ subunits liberated from the G proteins activated by the CaR may stimulate PI3'-kinase, which leads to activation of PKCζ. It should be noted that several of the enzymes involved in the postulated PI3'-kinase-mediated parallel signal transduction pathway do not require that [Ca²⁺]_i be elevated. Secretion, however, is a Ca²⁺-dependent phenomenon and is abolished by exposing PF cells to L-type Ca²⁺ channel blockers (McGehee et al., 1997). It is likely that Ca²⁺ is required for steps, such as docking and membrane fusion, which are downstream from those involved in the signal transduction cascade and may also be required for upstream or downstream steps that are still to be investigated.

REFERENCES

- Ashkenazi A, Peralta E, Winslow J, Ramachandran J, Capon D (1989) Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell* 487–493.
- Barasch JM, Mackey H, Tamir H, Nunez EA, Gershon MD (1987a) Induction of a neural phenotype in a serotonergic endocrine cell derived from the neural crest. *J Neurosci* 7:2874–2883.
- Barasch JM, Tamir H, Nunez EA, Gershon MD (1987b) Serotonin-storing secretory granules from thyroid parafollicular cells. *J Neurosci* 7:4017–4033.
- Barasch JM, Gershon MD, Nunez EA, Tamir H, Al-Awqati Q (1988) Thyrotropin induces the acidification of the secretory granules of parafollicular cells by increasing the chloride conductance of the granular membrane. *J Cell Biol* 107:2137–2148.
- Bernd P, Gershon MD, Nunez EA, Tamir H (1981) Separation of dissociated thyroid follicular and parafollicular cells: association of serotonin binding protein with parafollicular cells. *J Cell Biol* 88:499–508.
- Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC (1993) Cloning and characterization of an extracellular Ca²⁺-sensing receptor from bovine parathyroid. *Nature* 366:575–580.
- Carpenter C, Duckworth B, Auger K, Cohen B, Schaffhausen B, Cantley L (1990) Purification and characterization of phosphoinositide 3-kinase from rat liver. *J Biol Chem* 265:19704–19711.
- Chattopadhyay N, Legradi G, Bai M, Kifor O, Ye C, Vassilev P, Brown E, Lechan R (1997) Calcium-sensing receptor in rat hippocampus: a developmental study. *Dev Brain Res* 100:13–21.
- Cheng I, Qureshi I, Chattopadhyay N, Qureshi A, Butters R, Hall A, Cima R, Rogers K, Herbert S, Geibel J, Brown E, Soybel D (1999) Expression of an extracellular calcium-sensing receptor in rat stomach. *Gastroenterology* 116:118–126.
- Chou M, Hou W, Johnson J, Graham L, Lee M, Chen C, Newton A, Schaffhausen B, Toker A (1998) Regulation of protein kinase Cζ by PI3-kinase and PDK-1. *Curr Biol* 8:1069–1077.
- Cidon S, Tamir H, Nunez EA, Gershon MD (1991) ATP-dependent uptake of 5-hydroxytryptamine by secretory granules isolated from thyroid parafollicular cells. *J Biol Chem* 266:4392–4400.
- Clark M, Lanigan T, Page N, Russo A (1995) Induction of a serotonergic and neuronal phenotype in thyroid C-cells. *J Neurosci* 15:6167–6178.
- Cotecchia S, Daniel K, Kobilka B, Nolan R, Lapetina E, Caron M, Lefkowitz R, Regan J (1990) Multiple second messenger pathways of alpha-adrenergic receptor subtypes expressed in eukaryotic cells. *J Biol Chem* 265:63–69.
- Dell'Ackqua M, Carroll R, Peralta E (1993) Transfected m2 muscarinic acetylcholine receptors couple to Gαi2 and Gαi3 in chinese hamster ovary cells. *J Biol Chem* 268:5676–5685.
- Derman MP, Toker A, Hartwig JH, Spokes K, Falck JR, Chen CS, Cantley LC, Cantley LG (1997) The lipid products of phosphoinositide 3-kinase increase cell motility through protein kinase C. *J Biol Chem* 272:6465–6470.
- Emanuel R, Adler G, Kifor O, Quinn S, Fuller F, Krapcho K, Brown E (1996) Calcium-sensing receptor expression and regulation by extracellular calcium in AtT-20 pituitary cell line. *Mol Endocrinol* 10:555–565.
- Ettinger SL, Lauener RW, Duronio V (1996) Protein kinase Cδ specif-

- ically associates with phosphatidylinositol 3-kinase following cytokine stimulation. *J Biol Chem* 271:14514–14518.
- Feranchak A, Roman R, Doctor R, Salter K, Toker A, Fitz J (1999) The lipid products of phosphoinositide 3-kinase contribute to regulation of cholangiocyte ATP and chloride transport. *J Biol Chem* 274:30979–30986.
- Gomez J, Garcia A, Borlado L, Bonay P, Martinez C, Silva A, Fresno M, Carrera A, Eicher-Streiber C, Rebollo A (1997) IL-2 signaling controls actin organization through Rho-like protein family, phosphatidylinositol 3-kinase, and protein kinase ζ . *J Immunol* 158:1516–1522.
- Herbert SC, Brown EM (1995) The extracellular calcium receptor. *Curr Opin Cell Biol* 7:484–492.
- Herbert J, Augereau J, Gleye J, Maffrend J (1990) Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* 172:993–999.
- Herrera-Velitz P, Reiner N (1996) Bacterial lipopolysaccharide induces the association and coordinate activation of p53/56^{lyn} and phosphatidylinositol 3-kinase in human monocytes. *J Immunol* 156:1157–1165.
- Herrera-Velitz P, Knutson KL, Reiner NE (1997) Phosphatidylinositol 3-kinase-dependent activation of protein kinase C- ζ in bacterial lipopolysaccharide-treated human monocytes. *J Biol Chem* 272:16445–16452.
- Huntley C, De B, Banerjee A (1997) Phosphorylation of Sendai virus by cellular protein kinase ζ . *J Biol Chem* 272:16578–16584.
- Kapeller R, Cantley L (1994) Phosphoinositide 3-kinase. *BioEssays* 16:565–576.
- Kobayashi E, Nakano H (1989) Calphostin C, a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* 159:548–552.
- Kurosu H, Maehama T, Okada T, Yamamoto T, Hoshino S-I, Fukui Y, Ui M, Hazeki O, Katada T (1997) Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110 β is synergistically activated by the $\beta\gamma$ subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem* 272:24252–24256.
- Le Good J, Ziegler W, Parekh D, Alessi D, Cohen P, Parker P (1998) Protein kinase C isoforms controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 281:2042–2045.
- Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marmé D, Schächtele C (1993) Selective inhibition of protein kinase C isoforms by the indolcarbazole Gö 6976. *J Biol Chem* 268:9194–9197.
- Masayuki M, Tanabe Y, Tshuida K, Shigemoto R, Nakanishi S (1991) Sequence and expression of metabotropic glutamate receptor. *Nature* 349:760–765.
- McGehee D, Adlersberg M, Liu K, Hsiung SC, Heath M, Tamir H (1997) Mechanism of extracellular Ca²⁺ receptor-stimulated hormone release from sheep thyroid parafollicular cells. *J Physiol (Lond)* 502.1:31–44.
- Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science* 268:247–251.
- Nakanishi H, Brewer KA, Exton JH (1993) Activation of ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 268:13–16.
- Newton A (1997) Regulation of protein kinase C. *Curr Opin Cell Biol* 9:161–167.
- Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* 270:28495–28498.
- Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607–614.
- Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9:484–496.
- Nunez EA, Gershon MD (1978) Cytophysiology of thyroid parafollicular cells. *Int Rev Cytol* 52:1–80.
- Okada T, Sakuma L, Fukui Y, Hazeki O, Ui M (1994) Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J Biol Chem* 269:3563–3567.
- Ptasznik A, Prossnitz E, Yoshikawa D, Smrcka A, Trynor-Kaplan A, Bokoch G (1996) A tyrosine kinase signaling pathway accounts for the majority of phosphatidylinositol 3,4,5-trisphosphate formation in chemoattractant-stimulated human neutrophils. *J Biol Chem* 271:25204–25207.
- Rogers K, Dunn C, Herbert S, Edwards E (1997) Localization of calcium receptor mRNA in the adult rat central nervous system by in situ hybridization. *Brain Res* 744:47–56.
- Rordorf-Nikolic T, Van Horn D, Chen D, White M, Backer J (1995) Regulation of phosphatidylinositol 3'-kinase by tyrosyl phosphoproteins. *J Biol Chem* 266:3662–3666.
- Rotenberg S, Huang M, Zhu J, Sue L, Riedel H (1995) Deletion analysis of protein kinase C inactivation by calphostin C. *Mol Carcinog* 12:42–49.
- Ruat M, Snowman A, Hester L, Snyder S (1996) Cloned and expressed rat Ca²⁺-sensing receptor. *J Biol Chem* 271:5972–5975.
- Russo AF, Lanigan TM, Sullivan BE (1992) Neuronal properties of a thyroid C-cell line: partial repression by dexamethasone and retinoic acid. *Mol Endocrinol* 6:207–218.
- Sacktor T, Osten P, Valsamis H, Jiang X, Naik M, Sublette E (1993) Persistent activation of the ζ isoform of protein kinase C in the maintenance of long-term potentiation. *Proc Natl Acad Sci USA* 90:8342–8346.
- Sajan M, Standaert M, Bandyopadhyay G, Quon M, Burke T, Farese R (1999) Protein kinase C- ζ and phosphoinositide-dependent protein kinase-1 are required for insulin-induced activation of ERK in rat adipocytes. *J Biol Chem* 274:30495–30500.
- Shen S, Buck W (1990) A synthetic peptide of the pseudosubstrate domain of protein kinase C blocks cytoplasmic alkalization during activation of the sea urchin egg. *Dev Biol* 140:272–280.
- Shibasaki F, Homma Y, Takenawa T (1991) Two types of phosphoinositide 3-kinase: monomer and heteromer forms. *J Biol Chem* 266:8108–8114.
- Standaert M, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, Farese R (1997) Protein kinase C- ζ as a downstream effector of phosphatidylinositol 3-kinase in rat adipocytes. *J Biol Chem* 272:30075–30082.
- Tamir H, Liu KP, Hsiung SH, Adlersberg M, Nunez EA, Gershon MD (1990) Multiple signal transduction mechanisms leading to the secretion of 5-hydroxytryptamine by MTC cells, a neuroectodermally derived cell line. *J Neurosci* 10:3743–3753.
- Tamir H, Liu KP, Heath M, Adlersberg M, Gershon MD (1994a) Stimulus-induced secretion and vesicle acidification in serotonergic paraneurons (parafollicular cells): the role of voltage-gated Ca²⁺ channels. *Neurosci Abstr* 20:1719.
- Tamir H, Piscopo I, Liu K-p, Hsiung S, Adlersberg M, Nicolaidis M, Al-Awqati Q, Gershon MD (1994b) Secretagogue-induced gating of chloride channels in the secretory vesicles of a paraneuron. *Endocrinology* 135:2045–2057.
- Tamir H, Liu K-P, Adlersberg M, Hsiung S-C, Gershon MD (1996) Acidification of serotonin-containing secretory vesicles induced by a plasma membrane calcium receptor. *J Biol Chem* 271:6441–6450.
- Tang X, Downes C (1997) Purification and characterization of G $\beta\gamma$ -responsive phosphoinositide 3-kinase from pig platelet cytosol. *J Biol Chem* 272:14193–14199.
- Thomason PA, James SR, Casey PJ, Downes CP (1994) A G-protein $\beta\gamma$ subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. *J Biol Chem* 269:16525–16528.
- Toker A, Meyer M, Reddy K, Falck J, Aneja R, Aneja S, Parra A, Burns D, Ballas L, Cantley L (1994) Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J Biol Chem* 269:32358–32367.
- Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 269:5241–5248.
- Washburn D, Ferguson A (1998) Calcium receptor activation potentiates spike after-polarizations in rat subfornical organ neurons. *Soc Neurosci Abstr* 24:370.
- Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y (1993) Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J Biol Chem* 268:25846–25856.
- Zabel M (1984) Ultrastructural localization of calcitonin, somatostatin and serotonin in parafollicular cells of the rat thyroid. *Histochem J* 16:1265–1272.
- Zhou G, Seibenhener M, Wooten M (1997) Nucleolin is a protein kinase C- ζ substrate. *J Biol Chem* 272:31130–31137.