The Role of Protein Kinase C Isozymes in Bombesin-stimulated Gastrin Release from Human Antral Gastrin Cells*

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Two of the most effective stimuli of gastrin release from human antral G cells are bombesin and phorbol esters. Both agonists result in activation of the protein kinase C family of isozymes, however, the exact contribution of protein kinase C to the resultant release of gastrin has been difficult to assess, possibly due to the presence of multiple protein kinase C isozymes in the G cells. The results of the present study demonstrated that the human antral G cells expressed 6 protein kinase C isozymes α, γ, θ, e, ζ, and μ. Of these protein kinase C, γ and θ were translocated by stimulation of the cells by either 10 nM bombesin or 1 nM phorbol ester. Inhibition of protein kinase Cμ (localized to the Golgi complex) did not decrease bombesin-stimulated gastrin release indicating that this isozyme was not involved in the secretory process. The use of selective antagonists of the calcium-sensitive conventional protein kinase C subgroup resulted in an increase in bombesin-stimulated gastrin release and indicated that protein kinase Cy was involved in the desensitization of the bombesin response.

The protein kinase C (PKC) enzyme family represents a group of widely distributed serine/threonine kinases that play a variety of regulatory roles in cell signaling events (1). Twelve PKC isozymes, grouped into three major classes, have been identified to date: the conventional, cPKCs α, β (which exists in two splice variants βI and βII), and γ; the novel, nPKCs δ, ε, η, μ, and θ; and the atypical, aPKCs ζ and λ/ι (ι is the human homologue of the mouse isozyme λ). The latest isozyme identified, PKCμ, shares extensive sequence homologies with the newly described murine protein kinase D (PKD (2)). The cPKCs are activated by calcium and diacylglycerol (DAG), while the nPKCs, lacking C2 regions, require only DAG for activation. The last group, the aPKCs, with only one zinc finger motif in the C1 region and lacking C2 regions, are the least well characterized. The aPKCs are not activated by either calcium or DAG, but respond to phosphatidylinositol 3,4,5-triphosphate and arachidonic acid (1).

In endocrine cells a number of different PKC isozymes have been detected and linked to the regulation of hormone secretion. In normal rat pancreatic β cells and bovine parathyroid cells the predominant isozymes expressed are PKC α, β, and δ with detectable levels of ε and ζ but not γ (3). The pancreatic β cell line, MIN6, expressed the same isozymes as normal β cells with the addition of λ and μ (4). In thyroid cell lines PKC isoforms α, δ, ζ, and e were detected (5), while AtT20 cells express α, β, e, and ζ (6). It appears that the majority of endocrine cells share the expression of the isozymes α, e, and ζ but differ with respect to β, δ, λ, and μ. In functional studies activation of the isozymes α (β cells) and δ (thyroid cells) have been linked to hormone release (4, 5).

We have been investigating the signal transduction pathway activated by stimulation of human antral G cells by gastrin releasing peptide (GRP)/bombesin (BN). While we have strong evidence that activation of the GRP receptor results in the release of intracellular calcium from phosphatidylinositol 3,4,5-triphosphate-sensitive intracellular stores (7, 8), the role of PKC in BN-stimulated gastrin release has been more difficult to assess. However, phorbol esters are potent stimulators of gastrin release in this human antral cell preparation indicating that activation of DAG-sensitive isozymes can initiate gastrin containing secretory granule exocytosis (9).

The identification of the PKC family of enzymes suggests that multiple isozymes may be present in the G cells and inhibition of different isozymes could explain contradictory results obtained from the use of non-selective antagonists. In addition, activation of GRP receptors in the Swiss 3T3 fibroblast cell line stimulates activity of murine PKD (the homolog of human PKCμ) by a PKC-dependent mechanism (2). The precise PKC isozyme(s) responsible for stimulation of PKD activity has yet to be identified and it is unknown whether a similar effect occurs in our human cell preparation.

The present studies were designed first to determine which PKC isozymes were expressed by the antral G cells. Second to determine if the distribution of any of the isozymes could be altered by stimulation of the G cells with either 1 nM phorbol ester (PMA) or 10 nM BN, and finally to compare the ability of a series of different PKC antagonists to inhibit BN-stimulated gastrin release.

MATERIALS AND METHODS

Cell Isolation

Human antrum was obtained from 19 multiple organ donors in association with the British Columbia Transplant Society with ethical approval of the University of British Columbia Clinical Screening Committee. There were 11 males and 8 females the average age of the males was 28 years and the females 36 years. A single cell suspension of mucosal cells was prepared and separated by centrifugal elutriation as described previously (9). The F1 fraction containing the majority of the gastrin cells was used in subsequent experiments.

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‡ The abbreviations used are: PKC, protein kinase C; PKD, protein kinase D; DAG, diacylglycerol; GRP, gastrin releasing peptide; BN, bombesin; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcriptase-polymerase chain reaction.
All murine monoclonal antibody were from Transduction Laboratories.

| Antibody dilutions | Primary antibodies antigen | RCC | Western |
|---------------------|----------------------------|-----|---------|
| PKC α               | 1:50                       | 1:250 |         |
| PKC γ               | 1:500                      | 1:5,000 |       |
| PKC μ               | 1:100                      | 1:1,000 |       |
| PKC ε               | 1:50                       | 1:1,000 |       |
| PKC θ               | 1:100                      | 1:1,000 |       |
| PKC e               | 1:50                       | 1:500  |         |
| PKC ζ               | 1:50                       | 1:250  |         |

**Cell Culture**

The cells in the F1 fraction were resuspended at 1 × 10⁶ cells/ml in growth medium comprising: 50:50 Dulbecco's modified Eagle's medium/Ham's F-10, 1.0 mM Ca²⁺, 1 μg/ml hydrocortisone, 8 μg/ml insulin, 5% heat-inactivated fetal calf serum, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 10 μg/ml gentamycin. For immunocytochemical studies 1 ml/well was plated on 12-mm round glass coverslips pre-coated with 3-aminopropyltriethoxysilane solution in 24-well Costar plates. After 48 h in culture the cells were either washed in phosphate-buffered saline and fixed in 4% paraformaldehyde for 15 min at room temperature or stimulated with 1 nM PMA or 10 nM BN in the presence or absence of extracellular calcium prior to fixation.

To confirm the identity of the PKC isoforms detected by the antibodies in human antral cells, protein was extracted from the cultured cells using the Trizol reagent following the manufacturers (Life Technologies, Inc., Grand Island, NY) instructions. The antral proteins were separated proteins were transferred onto nitrocellulose membrane and nonspecific protein binding blocked by an overnight incubation in 2% milk powder in Tris-buffered saline with 0.05% Triton X-100 (TBST). The membrane was rinsed 3 times in TBST prior to incubation with one of the seven PKC isoyme-specific antisera employed for the Western blots diluted in TBST and 5% horse serum (for the relevant dilutions see Table I). After washing 3 times in TBST the membranes were incubated in horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:1000 in TBST for 1 h at room temperature. Protein bands detected by the antibodies were identified using the ECL Western blotting Detection system (Amersham Pharmacia Biotech, Piscataway, NJ) using Kodak diagnostic film processed in a Kodak M35A-OMAT processor.

**Immunocytochemistry**

To show the localization of actin in BN-stimulated and unstimulated G cells was determined by incubation of gastrin-immunostained coverslips with ALEXA 488-conjugated phalloidin (Molecular Probes, Eugene, OR) for 20 min at room temperature. Coverslips were washed extensively in phosphate-buffered saline prior to application of coverslips.

**Radioimmunossay**

The radioimmunossay for gastrin was performed using the CKG-polyclonal antibody as described previously (9). The assay detects the N-terminal region of human gastrin-17 and has a lower sensitivity limit of 5 fmol. Each sample was assayed in duplicate. The inter- and intra-assay variations were 10 and 5%, respectively.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

The primers were designed to amplify a portion of the mRNA spanning the variable regions 1 and 2 of the enzyme. The 5’ sequence CCGCGGCTAGGGCGATTCCAGA and reverse 3’ sequence TACGTTGATCTCATCTTGCTGT. Total RNA was isolated from human antral cells using Trizol (Life Technologies, Inc., Grand Island, NY) following the manufacturers’ directions. First strand cDNA was prepared from 3 μg of total RNA using Superscript II. The RNA was first incubated in first strand buffer (50 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, and 75 mM KCl), containing 32 units of RNasin (Pharmacia) and 1 unit of RNase-free DNase (Promega Corp., Madison, WI) for 45 min at 37 °C, followed by 75 °C for 10 min. A sample of this reaction was then used as a template in a subsequent PCR reaction. Random primers (400 pmol), dithiothreitol (to 10 mM) and 16 units of RNasin were added and the conjugated goat anti-rabbit IgG at a dilution of 1:1000 (Jackson Laboratories).

To establish if 1 nM PMA or 10 nM BN resulted in translocation of any of the PKC isoymes expressed in the gastrin cells, cells cultured on coverslips were stimulated for 1, 5, 15, or 30 min at 37 °C prior to fixation. The double staining protocol outlined above was completed and the localization of the isoymes in control or stimulated cells compared using digital imaging microscopy at each time point.

Finally the localization of actin in BN-stimulated and unstimulated G cells was determined by incubation of gastrin-immunostained coverslips with ALEXA 488-conjugated phalloidin (Molecular Probes, Eugene, OR) for 20 min at room temperature. Coverslips were washed extensively in phosphate-buffered saline prior to application of coverslips.

**Digitial Imaging Microscopy—Cells were examined on the stage of a Nikon Diaphot 200 inverted microscope in epifluorescent mode, with a UV-F 100/1.3 glycerin immersion objective and narrow band-pass filters from Omega Optical for fluorescein isothiocyanate and tetramethyl-rhodamine isothiocyanate (Brattleboro, VT). A series of two-dimensional images were acquired of the cells, through focus, at 0.25-μm intervals. The camera was a Photometrics 200 equipped with a thermoelectrically cooled, back-illuminated, Tektronix TK512CB chip (Tucson, AZ). Voxel dimensions were 122 nm × 122 nm × 250 nm. Image stacks were transferred to a Silicon Graphics Indigo™ WX workstation where they were dark-current and background subtracted, and corrected for non-uniformities across the field of view and illumination. For every day on which images were acquired, the optical transfer function of the microscope was empirically measured using fluorescein-taged beads (100 nm diameter) obtained from Molecular Probes. The measured optical transfer function was then used to deconvolve the data sets using a constrained, iterative, deconvolution algorithm based on regularization theory (10). Deconvolutions were performed on a Scinetics EPR server (Bellericia, MA). Pairs of deconvolved images were then aligned using small fluorescent beads, with broad excitation and emission maxima, which had been included in the mounting medium as fiducary markers. The intensity of a small region of each deconvolved image (5 × 5 × 3 voxels) was selected as representative of the background intensity of that image and used as the threshold for non-specific deconvolution of wide field images, rather than confocal microscopy, was selected for this phase of the work since for discretely organized objects from which the emitted fluorescence intensity is low, deconvolving wide field images produces results superior to those that can be obtained from confocal microscopes (11).
Fig. 1. A, unstimulated cells immunostained with the PKC\(\theta\) antibody. Note the concentration of immunoreactivity at the plasma membrane (arrows). B, the same cell cluster immunostained for gastrin demonstrating that PKC\(\theta\) immunoreactivity was restricted to the G cells (arrows). C, unstimulated cells immunostained for PKC. Note that the immunoreactivity is concentrated at the plasma membrane of non-G cells (small arrows). D, the same group of cells immunostained for gastrin showing a PKC\(\iota\) negative cell (large arrow). E, a group of unstimulated cells immunostained using the PKC\(\iota\) antibody. Note that only the G cell (arrow) shows a small group of immunoreactive vesicles. F, the same group of cells immunostained using the gastrin antibody. Note that the PKCs immunoreactivity was confined to the G cell (arrow). G, an unstimulated group of cells immunostained using the PKC\(\iota\) antibody. Note that the immunoreactivity is limited to a cluster of vesicles around the nucleus of gastrin-immunoreactive cells (arrow). H, the same group of cells immunostained for gastrin, note that the PKC\(\iota\) immunoreactivity is limited to the G cells (arrow). I, a group of cells immunostained by the PKC\(\iota\) antibody. Note that the immunoreactivity appears to be associated with small vesicles distributed throughout the cytoplasm. J, the same group of cells immunostained for gastrin demonstrating that gastrin immunoreactivity was concentrated to one end of the cells while PKC\(\alpha\) immunoreactivity was present throughout the cells. K, a group of cells immunostained using the PKC\(\gamma\) antibody. Note that one of the cells is a G cell (large arrow) the other is a non-gastrin (somatostatin) cell (small arrow). L, the same group of cells immunostained for gastrin showing the gastrin immunoreactive cell (large arrow). M, a group of cells immunostained with the PKC\(\mu\) antibody showing that the immunostaining is confined to the Golgi complex of all cells in the cultures. N, the same group of cells immunostained for gastrin. Note the immunostaining of the Golgi complex (arrows) is distinct from the localization of the gastrin immunoreactive secretory granules. All scale bars represent 10 \(\mu\)m.

### RESULTS

**Immunocytochemistry**

**Protein Kinase C Isozymes**—To establish which PKC isozymes were expressed in the cultured antral cells single immunostainings were completed. These demonstrated the presence of PKC isozymes \(\alpha\), \(\gamma\), \(\epsilon\), \(\zeta\), \(\iota\), \(\theta\), and \(\mu\). Their location in unstimulated cells was isozyme specific: \(\theta\) and \(\iota\) were associated with the plasma membrane (Fig. 1 A and C), while \(\epsilon\) and \(\zeta\) were localized to intracellular vesicles (Fig. 1, E and G), \(\alpha\) and \(\gamma\) were present throughout the cytoplasm (Fig. 1, I and K) while \(\mu\) was confined to the Golgi complex (Fig. 1M). Double immunostaining demonstrated that isozymes \(\theta\), \(\epsilon\), \(\zeta\), \(\alpha\), and \(\gamma\) were expressed in the gastrin cells (Fig. 1, B, F, H, J, and L), \(\iota\) was expressed in non-gastrin cells (Fig. 1D) and \(\mu\) was expressed in all the cultured cells.
Translocation Experiments—The major focus of the present studies was to determine which PKC isozymes were involved in BN-stimulated gastrin release. Once the isozymes expressed in the G cells had been established the next series of experiments evaluated if BN or PMA treatment caused the translocation of specific isozymes. Stimulation of the G cells with 10 nM BN resulted in the translocation of PKCγ from a predominantly intracellular localization to the plasma membrane (Fig. 2, A and B). Interestingly, although somatostatin cells in the culture preparation also contain PKCγ the location of the isozyme in these cells was not affected by BN treatment (see Fig. 2). These data indicated the specificity of the translocatory effect to the gastrin cells known to express the GRP receptor. The translocation occurred within 1 min of the addition of BN and within 5 min PKCγ had returned to the cytosol.

In addition to altering the intracellular distribution of PKC isoforms the morphological appearance of the G cells was significantly modified by BN treatment. The increased resolution and sensitivity of wide field microscopy coupled with the use of a deconvolution algorithm and image analysis was utilized to examine these alterations. Within 1 min of addition of BN the gastrin cells developed structures resembling lamellipodia on their lateral margins (Fig. 3, B and C). The lamellipodia were outlined by PKCγ immunoreactivity but did not contain gastrin-immunoreactive secretory granules. Addition of 1 nM PMA also resulted in translocation of PKCγ at all time points examined, however, no lamellipodia-like structures were observed (Fig. 3D).

Previous studies had indicated that activation of GRP receptors with BN resulted in the activation of PKD in Swiss 3T3 cells (2). In the human cell preparation, PKCμ (the human homolog of PKD) was localized to the Golgi complex of all the cells. Deconvolution of wide field images was used to determine if BN or PMA treatment altered the distribution of this isozyme specifically in the gastrin cells. The immunostainings demonstrated that neither BN nor PMA had any effect on the distribution of this isozyme (Fig. 2). In the human cell preparation, PKCμ was localized to the Golgi complex of all the cells (2). In the human cell preparation, PKCμ was localized to the Golgi complex of all the cells.

Fig. 2. A, two cells immunostained using the antibody to PKCγ. Note that only the G cell (small arrow) shows translocation of the protein to the cell membrane. B, the same cell group immunostained for gastrin. Note that the lower cell shows no gastrin immunoreactivity (large arrow). Scale bar, 10 μm.

With the PKD isoform, the small arrow shows translocation to the plasma membrane. In addition, the larger arrow shows that the protein is localized to the Golgi complex.

Western Blots—Of the seven antibodies used for the Western blot analysis five gave positive results showing bands of the expected size (Fig. 5). The exceptions were PKCε and α where no band was detected in the antral cells. This was most probably due to the low levels of the isozymes expressed in the cells as determined by the immunocytochemical results. In addition to bands of the expected size the Western blots for PKCμ, θ, and ε showed additional bands at smaller molecular weights consistent with degredation of the mature protein. The Western blot for one isozyme, PKCδ, showed a band of a larger size than the control band, however, the control protein was obtained from rat tissue and the human protein may be of a larger molecular weight.

Reverse Transcription-Polymerase Chain Reaction—In view of the lack of expression of PKCγ outside the nervous system we confirmed the expression of the isozyme by RT-PCR. Primers specific to PKCγ amplified a band of the expected size by RT-PCR but not from a negative control (Fig. 6). Subsequent sequence analysis confirmed that the amplified DNA was part of the sequence of the PKCγ gene.

Release Experiments

In all experiments BN addition resulted in a concentration-dependent increase in gastrin release (n = 16). Addition of 1 μM staurosporine, a broad spectrum inhibitor of protein kinases, resulted in a significant inhibition of BN-stimulated gastrin release (Fig. 7). The structurally similar compound, bisindolylmaleimide, that acts as a competitive inhibitor for the ATP-binding site of PKC and is relatively selective for the isozymes α, β, γ, δ, and ε was added to the medium at 1 μM 15 min prior to and during stimulation with BN. This compound resulted in a significant increase in BN-stimulated gastrin release at 0.1–1 nM but not at the highest concentration of BN (10 nM) (Fig. 8A).

The final two compounds in this group were Go 6983 and Go 6976, again structurally similar to staurosporine, but are selective inhibitors of different PKC isozymes. The more selective is Go 6976 that at nanomolar concentrations inhibits isozymes α, β, γ, and μ whereas, nanomolar concentrations of Go 6983 inhibit α, β, γ, δ, and ε. Addition of either 10 nM Go6976 or 100 nM Go6983 resulted in a significant stimulation of BN-stimulated gastrin release (Fig. 8B).

The plant alkaloid, chelerythrine chloride, does not have a documented selectivity for individual isozymes but is a compet-
itive inhibitor at the phosphate acceptor site of PKCs and a noncompetitive inhibitor at the ATP-binding site. Addition of 100 nM chelerythrine chloride both 15 min before and during stimulation with BN had no effect on gastrin release at any BN concentration tested (data not shown).

Calphostin C is structurally distinct from the first group of PKC antagonists and competes for binding at the diacylglycerol/phorbol ester-binding site of the enzymes. This compound will not affect the aPKCs and requires light for activation. In order to complete these experiments the culture plates were removed from the incubator and maintained on a 37 °C heated stage in a humid plexiglass chamber for the 1-h incubation period. Addition of 100 nM calphostin C resulted in a significant inhibition of BN-stimulated gastrin release at the higher concentrations (Fig. 9).

**DISCUSSION**

Immunocytochemical staining identified the presence of 6 distinct PKC isoymes in human antral G cells: α, γ, θ, ε, ζ, and μ. Each isozyme demonstrated a different localization within

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**Fig. 3.** The images are three-dimensional reconstructions of the deconvolved, aligned, and thresholded image pairs. Gastrin granules are pseudocolored green, and the indicated PKC isoform is pseudocolored red. If both proteins occupied the same voxel, then that voxel became white. For display of the three-dimensional image, the opacity of a given voxel is directly proportional to its intensity. Scale bar is 2.44 μm. A, in an unstimulated cell PKCγ immunoreactivity was restricted to a population of randomly distributed vesicles within the cytoplasm (red). These vesicles showed a minimal overlap with the gastrin-immunoreactive secretory granules (green). B, a gastrin cell 1 min after stimulation with 10 nM BN. Note that the majority of the PKCγ immunoreactivity is now associated with the plasma membrane and the presence of membrane protrusions (arrows). C, 5 min after stimulation with BN some PKCγ immunoreactivity remains at the plasma membrane associated with membrane protrusions (arrows). D, an example of PKCγ immunostaining in a G cell 15 min after stimulation with 1 nM PMA. Note that the majority of the PKCγ immunoreactivity is still associated with the plasma membrane and the lack of membrane protrusions. E, in an unstimulated G cell PKCμ immunoreactivity was limited to the Golgi complex with minimal overlap with the gastrin-immunoreactive secretory granules. F, in a PMA-stimulated cell, PKCμ immunoreactivity remains confined to the Golgi complex and shows minimal overlap with gastrin-immunoreactive secretory granules. G, an unstimulated gastrin cell immunostained for PKCθ. Note that patchy immunoreactivity for PKCθ is associated with the plasma membrane in the apical region (arrow). In addition, there is little overlap with the gastrin-immunoreactive secretory granules. H, after a 5-min stimulation with 1 nM PMA the immunoreactivity for PKCθ covers regions of the plasma membrane directly overlying gastrin immunoreactive secretory granules at the basal membrane of the G cell. In addition, PKCθ is concentrated to a ring around the apical zone of the cell (arrow). I, after a 10-min incubation in 1 nM PMA PKCθ immunoreactivity appears to be concentrated over the microvilli at the apical pole of the G cell (arrow). In addition, there in an increased overlap of PKCθ and gastrin immunoreactivity at the basal pole of the cell (white vesicles).
the G cells. The two cPKC isozymes α and γ were diffusely distributed throughout the cytoplasm of unstimulated cells with PKCγ being the more abundant. No evidence was found for the presence of the third cPKC, β, although the antibody was capable of detecting the protein in rat cardiomyocytes fixed using an identical protocol.

The expression of PKCγ has been thought to be limited to the nervous system, therefore, we confirmed the immunocytochemical results using RT-PCR and Western blot analysis. Both additional techniques confirmed that the antral cells expressed the isozyme.

If either cPKC were to be involved with the regulation of gastrin secretion we expected that stimulation of the cells with BN or PMA would result in a translocation of the active isozyme from an intracellular location to the cell membrane. Stimulation of the G cells with both agonists resulted in a translocation of PKCγ from a predominantly intracellular location to the plasma membrane. The time course of the BN-stimulated translocation paralleled that previously determined for the increase in intracellular calcium in the G cells after stimulation with BN (8). The parallel time course of these events indicates that the mobilization of intracellular calcium by activation of the GRP receptor provided the calcium required to activate PKCγ. The rapidity of the PKC translocation was similar to that reported in HEK cells transfected with a green fluorescent protein-PKCβ (GFP-PKCβ) construct and the green fluorescent protein-tagged N-terminal cysteine-rich region of PKCγ (12, 13).

When G cells were stimulated with BN (but not PMA) the cells developed protrusions at both lateral and basal surfaces. These structures resemble lamellipodia that are specialized membrane regions known to be generated in cells after stimulation of the Rac signaling pathway (14). In the Swiss 3T3 cell line stimulation of GRP/BN receptors with BN results in activation of the Rac signaling pathway resulting in the reorganization of the actin cytoskeleton and the generation of lamellipodia (15).

In antral cells stained using fluorescent phalloidin we observed a thin cortical layer of actin underlying the plasma membrane with no stress fibers or lamellipodia. In stimulated cells the cortical actin showed distinct gaps at the basal pole of the cells underlying the secretory granules, whereas regions at the lateral sides of the cells showed accumulations of actin. The accumulation of actin in these regions is consistent with the formation of lamellipodia. Interestingly, 1 min after addition of BN these structures could be identified in the G cells by the concentration of PKCγ immunoreactivity in the presumptive lamellipodia indicating that this isozyme may be involved in their formation.

Three nPKC isozymes were localized to the G cells, PKCe, θ, and μ. However, their distribution within the cells was quite distinct. The ε isozyme was associated with a small group of

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2 E. D. W. Moore, unpublished data.
vesicular structures adjacent to the nucleus and this distribution was not affected by stimulation of the cells by either BN or PMA. Depending on the cell type investigated the reported intracellular localization of PKC\(\varepsilon\) differs. In NIH 3T3 cells overexpressing epitope-tagged PKC\(\varepsilon\) the isozyme was localized to the Golgi complex (16). Whereas, in hippocampal neurons and gastric parietal cells PKC\(\varepsilon\) was associated with filamentous actin (17, 18). Finally in pancreatic \(\beta\) cells PKC\(\varepsilon\) had a predominantly cytosolic location in unstimulated cells relocating to the plasma membrane after stimulation with glucose (19). The localization of the isozyme in the antral gastrin cells did not correspond with any of those previously reported indicating that the intracellular distribution of PKC\(\varepsilon\) is cell-specific.

The second nPKC, \(\theta\), was clearly localized to the plasma membrane of unstimulated G cells and did not overlap with gastrin-immunoreactive secretory granules. In cells stimulated with PMA, PKC\(\theta\) immunoreactivity was found to concentrate in regions overlying the gastrin-immunoreactive secretory granules and at the apical pole of the G cells. In the human lung carcinoma cell line, A549, stimulation of the cells with 25 nM PMA resulted in the translocation of PKC\(\theta\) from the cytosol to the cell membrane and nucleus (20). In the present study we have no evidence for the presence of PKC\(\theta\) in the nucleus of either control or stimulated cells.

The significance of the translocation of PKC\(\theta\) to the apical region of the plasma membrane in the PMA stimulated cells is uncertain, however, PKC plays a role in the phosphorylation of proteins associated with the tight junction complex at the apical region of epithelial cells (21). The pattern of immunostaining observed with the PKC\(\theta\) antibody was distinct from that seen using an antibody to the tight junction protein, ZO-1, that forms a ring around the apical membrane of the cultured cells.\(^3\) In the majority of G cells the immunoreactivity for PKC\(\theta\) in the PMA-stimulated cells appeared to be associated with the plasma membrane of the microvilli at the apical region, rather than being limited to the tight junction zone itself. Whether this translocation has any involvement in PMA-stimulated gastrin secretion cannot be determined in the absence of specific isozyme antagonists.

The final nPKC, \(\mu\), was clearly localized to the Golgi compartment of all of the cultured cells confirming earlier studies in the human hepatocellular carcinoma cell line, HepG2 (22). The circumscribed distribution of PKC\(\mu\) suggests that it may be important in the processing of progastrin in the Golgi stack. Analysis of the structure of PKC\(\mu\) has demonstrated the presence of a PH-domain capable of binding the \(\beta\gamma\) subunit complex of heterotrimeric G proteins (22). This fact, coupled with the ability of \(\beta\gamma\) subunits to regulate vesicular transport processes in the Golgi complex, implies that PKC\(\mu\) may be involved in mediating the transport of proteins through the Golgi stack.

Previous studies of the activation of GRP receptors in Swiss

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\(^3\) A. M. J. Buchan unpublished data.
3T3 fibroblasts have demonstrated a PKC-dependent phosphorylation of murine PKD, a homolog of human PKC\(\mu\) (2). In the present study we obtained no evidence indicating that the location of PKC\(m\) in the Golgi complex could be altered by stimulation of the cells by either BN or PMA nor was there an appreciable overlap between gastrin- and PKC\(\mu\)-immunoreactive structures. These results indicated that while stimulation of the GRP receptor in the G cells may activate PKC\(m\) this was unlikely to influence exocytosis of gastrin containing secretory granules.

The only atypical PKC isoform localized to the gastrin cells was PKC\(\zeta\). This isoform was localized to perinuclear vesicles that were clearly distinct from the gastrin-immunoreactive secretory granules. The localization of this isoform was not affected by addition of either BN or PMA to the cell cultures and indicated that PKC\(\zeta\) was unlikely to be involved in the exocytotic process.

The morphological studies established which PKC isoforms were present in the gastrin cells but could not determine if any of the isoforms were directly involved in the secretory response. To evaluate if any of the PKC isoforms localized to the G cells played a role in BN-stimulated gastrin release the effect of a number of different PKC antagonists was examined. The majority of these antagonists are based on the sequence of the broad spectrum kinase inhibitor, staurosporine, and are directed to the ATP-binding site of the enzymes (23). Modifications to the basic structure of staurosporine have produced a number of antagonists with increasing isozyme specificity (24–26).

Addition of staurosporine itself decreased BN-stimulated gastrin release at all concentrations investigated, indicating that activation of PKC was capable of stimulating secretory granule exocytosis. However, staurosporine did not return BN-stimulated gastrin release to basal levels suggesting that additional signaling pathways were activated by the GRP receptor. These data coupled with the fact that the morphological studies demonstrated the presence of lamellipodia-like structures suggests that activation of the Rac signaling pathway may contribute to the observed gastrin secretion. The structurally related compounds, bisindolylmaleimide 1, Go 6976, and Go 6983, inhibitors of c- and nPKCs (24–26), all resulted in a significant increase in BN-stimulated gastrin release, although in parallel experiments PMA-stimulated gastrin release was inhibited as expected.4

Previous studies in Swiss 3T3 and CHO-K1 cells have demonstrated that the GRP receptor is phosphorylated on Ser and Thr residues after the addition of either BN or PMA (27, 28). Inhibition of c- and nPKCs by either PMA-mediated down-regulation or addition of bisindolylmaleimide resulted in an increased phosphorylation of the receptor on Ser and Thr residues after ligand (BN) binding. In the present studies inhibition of cPKCs resulted in a significant stimulation of gastrin release thus it is unlikely that additional Ser/Thr kinases capable of phosphorylating the BN receptor were activated in the G cells. If such enzymes were present bisindolylmaleimide would have resulted in increased BN receptor phosphorylation resulting in desensitization of the receptor and an inhibition of gastrin release.

Of the cPKC isoforms expressed in the antral cell cultures only PKC\(\gamma\) was translocated to the plasma membrane after BN stimulation. The time course of this translocation paralleled that reported for phosphorylation of the GRP/BN receptor. Translocation was detectable after 1 min and PKC\(\gamma\) remained associated with the membrane for up to 5 min after addition of BN. Receptor phosphorylation in the cell lines was detected 2 min after BN addition (26, 27). These data imply that PKC\(\gamma\) in the antral G cells was involved in phosphorylation and desensitization of the BN receptor.

The results of the experiments with Go 6983 and 6976 also support the lack of importance of PKC\(\mu\) in BN-stimulated gastrin release. These two inhibitors can be used to isolate effects due to activation of PKC\(\mu\) as Go 6976 inhibits the isozyme with an IC\(_{50}\) of 10 nM whereas Go 6983 is ineffective at nanomolar concentrations (26). In the present study inhibition of PKC isoforms with both compounds resulted in an increase in gastrin release, if PKC\(\mu\) activation was involved in the exocytotic process treatment with Go 6976 should have had an inhibitory effect.

Apart from staurosporine, calphostin C, a chemically unrelated compound that blocks the DAG-binding site (29, 30), was the only PKC antagonist that inhibited BN-stimulated gastrin release. Of the isoforms expressed in the G cells not inhibited by bisindolylmaleimide and related compounds, calphostin C should inhibit PKC\(\theta\) but not the nPKC, \(\zeta\). The fact that inhibition of cPKCs resulted in a stimulation of gastrin release suggested that inactivation of the nPKC, \(\theta\), was responsible for the observed decrease in gastrin release in the presence of calphostin C (and possibly staurosporine).

The data obtained during the present study demonstrated that at least 6 PKC isoforms are expressed in the antral G cells. Of these only PKC\(\gamma\) and \(\theta\) showed marked translocation in gastrin cells stimulated by either BN or PMA. The precise role of PKC\(\theta\) in BN-stimulated gastrin release cannot be determined due to the lack of specific inhibitors of the novel PKC isoforms. Inhibition of cPKC isoforms resulted in an increase in BN-stimulated gastrin release implicating PKC\(\gamma\) in the reported down-regulation of GRP receptor function by PKC. The confined localization of PKC\(m\) to the Golgi complex and the fact that its inhibition failed to decrease BN-stimulated gastrin release indicated that this isoform was not involved in secretory granule exocytosis.

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