Differential activation of phosphoinositide 3-kinase by endothelin and ceramide in colonic smooth muscle cells

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Su, Xuehui, Pinglang Wang, Adenike Ibitayo, and Khalil N. Bitar. Differential activation of phosphoinositide 3-kinase by endothelin and ceramide in colonic smooth muscle cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G853–G861, 1999.—We have investigated the hypothesis that different contractile agonists activate distinct catalytic subunits of phosphoinositide (PI) 3-kinase in smooth muscle cells. Endothelin (10⁻⁷ M) induced a sustained increase in PI 3-kinase activity at both 30 s and 4 min of stimulation (151.5 ± 8.5% at 30 s and 175.8 ± 8.7% at 4 min, P < 0.005). Preincubation of smooth muscle cells with the tyrosine kinase inhibitor genistein (3 µM) resulted in a significant inhibition of both C₂ ceramide-induced and endothelin-induced PI 3-kinase activation and contraction. Preincubation with herbimycin A, an Src kinase inhibitor (3 µM), inhibited only C₂ ceramide-induced PI 3-kinase activation and contraction. Western blotting using Src kinase antibody showed that C₂ ceramide, not endothelin, stimulated the phosphorylation of Src kinase. Western blotting and immunoprecipitation with PI 3-kinase antibodies to the regulatory subunit p85 and the catalytic subunits p110α and p110γ indicated that both endothelin and C₂ ceramide interacted with the regulatory subunit p85; endothelin interacted with the catalytic subunits p110α and p110γ, whereas C₂ ceramide interacted only with the catalytic subunit p110α. In summary, C₂ ceramide activated PI 3-kinase p110α subunit by a tyrosine kinase-mediated pathway, whereas endothelin-induced contraction, unlike C₂ ceramide, was not mediated by the activation of Src kinase but was mediated by G protein activation of both p110α and p110γ subunits (type IA and IB) of PI 3-kinase.

Src kinase; G proteins; tyrosine kinase

PHOSPHOINOSITIDE (PI) 3-kinase is a cytosolic enzyme that plays key roles in mediating signaling, including receptor-stimulated mitogenesis, oxidative burst, membrane ruffling, and glucose uptake (17). The activation of PI 3-kinase results in an increase in cellular levels of D-3 phosphorylated phosphoinositides, such as phosphatidylinositol 3-phosphate (PI-3-P), phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate, which have been proposed to act as second messengers (11, 14, 27).

PI 3-kinase is activated by a variety of growth factors, oncogenes, and other cellular activators (3). Most of the factors that activate this enzyme do so by turning on a cellular protein tyrosine kinase. PI 3-kinase is activated by platelet-derived growth factor, colony-stimulating factor-1, insulin, hepatocyte growth factor, epidermal growth factor, and interleukin-2 (15).

The receptors of all these factors either have intrinsic protein tyrosine kinase activity or activate associated tyrosine kinase of the Src family. In most of these cases, the activation of PI 3-kinase correlates with the recruitment of this enzyme from the cytosolic fraction to an activated protein tyrosine kinase (3). Thrombin and formyl peptides appear to activate PI 3-kinase via G protein-linked pathways (8, 28). In our current study, two contractile agonists (endothelin and C₂ ceramide) were found to activate PI 3-kinase via different pathways, leading to contraction of colonic smooth muscle cells.

The heterodimeric form of PI 3-kinase is made up of a regulatory subunit, p85, and a catalytic subunit, p110. At least two types of PI 3-kinase, in terms of the mode of activation, have been described in mammalian cells. They are type IA, which is stimulated by membrane-bound receptors activating tyrosine kinase, and type IB, which is under direct control of the heterotrimeric GTP-binding proteins (18). They have been structurally characterized as a heterodimer consisting of 110-kDa catalytic subunits (p110α, p110γ) and 85-kDa regulatory subunits (p85α, p85γ; see Ref. 30). Stimulation of tyrosine kinase receptors by extracellular signals phosphorylates specific tyrosine residues located in the YMXM motifs of their own receptors or adaptor molecules, such as insulin receptor substrate-1. These phosphorylated proteins bind to the SH2 domains of p85 and stimulate the lipid kinase activity (18). Type IB PI 3-kinase is activated by α- and βγ-subunits of G proteins; the adaptor is unknown, and the catalytic subunit p110γ may or may not be associated with the p85 adaptor (14). Recently, it was found that p110γ was associated with a noncatalytic p101 subunit (20). Several lines of evidence indicate that G protein stimulates p110γ in the absence of p101 both in vitro and in vivo (9, 10, 12, 22, 24). Gβγ are thought to be the dominant physiological stimulants, whereas Gα subunits of the G₁ but not Gα or G12 subfamilies only moderately activate p110γ (11).

Our previous study on C₂ ceramide (an intracellular product of sphingomyelin hydrolysis; see Ref. 6) set up a model showing that tyrosine kinase and Src kinase are upstream of PI 3-kinase, leading to smooth muscle contraction. We have used endothelin-1 (a potent vasconstrictor) and C₂ ceramide (a contractile agonist) on colon smooth muscle cells and found that C₂ ceramide activated the PI 3-kinase p110α subunit via activation of tyrosine kinase and activation of Src kinase pathways, whereas endothelin activated the PI 3-kinase p110γ subunit mainly through a different pathway, most likely through the G protein-mediated pathway (2, 14). C₂ ceramide activated type IA PI 3-kinase, and endothelin activated both type IA and type IB PI 3-kinase.
MATERIALS AND METHODS

Materials

The following reagents were purchased. Collagenase type II was purchased from Worthington Biochemical (Freehold, NJ); DMEM and genistein were from Life Technologies (Gaithersburg, MD); endothelin-1 was from Peninsula Laboratories (Belmont, CA); C2 ceramide was from Matreya (Pleasant Gap, PA); G protein-Sepharose was from Pharmacia Biotech (Piscataway, NJ); protein assay standard and goat anti-mouse IgG (heavy and light chains) horseradish peroxidase conjugate were from Bio-Rad (Hercules, CA); phosphotyrosine-specific antibody, PI 3-kinase p110α antibody, and p110γ antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); PI 3-kinase p85 antibody was from Upstate Biotechnology (Lake Placid, NY); anti-Src (P416Y) antibody (gift sample) was from New England Biolabs (Beverly, MA); [γ-32P]ATP, enhanced chemiluminescence detection reagents, and high-performance autoradiography film were obtained from Amersham (Arlington Heights, IL); and analytical silica gel 60 precoated glass TLC plates were from Merck (Darmstadt, Germany). All other reagents were purchased from Sigma (St. Louis, MO).

Methods

Isolation of smooth muscle cells from rabbit rectosigmoid.

New Zealand White rabbits were killed, and their internal sphincter consisting of the distal 3 mm of the circular muscle layer and ending at the junction of skin and mucosa were removed by sharp dissection. A 5-cm length of the rectosigmoid orad to the junction was dissected and digested to yield isolated smooth muscle cells. Cells were isolated as previously described (2). The tissue was incubated for two successive 60-min periods at 31°C in 15 ml of HEPES buffer containing 0.1% collagenase (150 U/mg; Worthington CLS type II) and 0.01% soybean trypsin inhibitor. After the second enzymatic incubation period, the medium was filtered through 500 μm Nitek mesh. The partially digested tissue left on the filter was washed four times with 50 ml of collagenase-free buffer solution. Tissue was then transferred into 15 ml of collagenase-free buffer solution and was incubated for 30 min on a Crickert with 1 ml of 100 mM Tris-buffered saline (TBS) buffer (0.1 M Tris and 0.154 M NaCl, pH 7.5) and rocking for 5 min. At the end of the last washing, the precipitates were resuspended, and 30 μl of PI 3-kinase buffer (20 mM Tris base, pH 7.4, and 10 mM MgCl2) were added. The precipitates were kept on ice before use.

PI 3-kinase activity determination.

The PI 3-kinase activity technique was based on previously described techniques (10, 19, 31) with some modification. The details were as follows: substrates were prepared by extracting the lipid mixture of 10 μg of phosphatidylinerine and 10 μg of phosphatidylinositol in 1 ml of methanol-chloroform (1:1) followed by sonication in 10 μl of PI 3-kinase buffer. The kinase reaction included 15 μl of kinase buffer, 10 μl of lipid substrate mixture, and 10 μl of kinase-containing beads. The reaction was set at 30°C for 10 min beginning by adding 10 μl of 20 μCi [γ-32P]ATP (2 μl labeled ATP in 8 μl of 104 μM ATP buffer) with 20 μCi radioactivity to phosphorylate the hydroxyl group of the substrate PI. The product was PI-3-P. Phosphatidylserine worked as a lipid carrier to ease the reaction. Each sample reaction was done in duplicate, and a negative control was carried out by replacing the substrates with the same volume of PI 3-kinase. The reaction was stopped by adding 100 μl of 1 N HCl. The lipid product was extracted in 160 μl of methanol-chloroform (1:1, vol/vol). The product was resolved by TLC using CHCl3-MeOH-NH4OH-d2H2O (45:35:1.5:8.5, vol/vol/vol/vol). PI-3-P was used as standard, and 5 μl were loaded on the plate. Sample products were loaded at 40 μl each. After the chromatographic development was finished, TLC plates were dried and placed in a tank for 10 min filled with one spoon of solid iodine. The iodine was used as an indicator to show the position of the product. Finally, the PI-3-P region was densitometrically quantitated [units: optical density (OD)·mm 2] after autoradiography. The autoradiography and densitometry were performed with a Bio-Rad Molecular Imager and the Quantity One 1.2 statistical program using ANOVA and analogous parametric tests (Student’s t-test, unpaired test, two-tailed P value).

Immunoprecipitation with mouse monoclonal phosphotyrosine antibody and Western immunoblotting using either a monoclonal IgG anti-Src (pp60src) antibody, a mouse monoclonal PI 3-kinase p85 antibody, a polyclonal PI 3-kinase p110α antibody, or a polyclonal PI 3-kinase p110γ antibody. Smooth muscle cells were counted on a hemocytometer and were diluted with HEPES buffer as needed. Cells were then treated with reagents for indicated periods. After treatment, the cells were washed with PBS and then disrupted by sonication in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na3VO4, 1 mM NaF, 1 mM Na3P04, 1 mM DTT, 20 mM Na2HPO4·21H2O, 50 μg/ml DNase/RNase A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA). Protein concentration was adjusted to 0.5–1 μg/μl, with the total protein amount at 400 μg. Anti-phosphotyrosine antibody was added to each sample (20 μl/250 μg protein) and was rocked for 1.5 h followed by 2 h rocking after addition of 40 μl G protein-Sepharose. Samples were centrifuged at 14,000 rpm. The precipitate (100 μg protein) was subjected to SDS-PAGE (7.5% gel) and electrophoretically transferred to an Immob-
ion P transfer membrane. The membrane was incubated with 5% skimmed milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature and was washed with TBST. The filter was incubated with the primary antibody (either anti-p85 or anti-Src at 1:500 and either anti-p110α or anti-p110γ at 1:200) in TBST-milk for 1 h at room temperature. After being washed with TBST (two quick washings, 2 × 5 min then 2 × 15 min), the filter was incubated with horseradish peroxidase anti-mouse IgG antibody at 1:5,000 in TBST-milk for 1 h at room temperature. After being washed with TBS (without Tween 20), the enzymes on the filter were visualized with luminescent substrates using an enhanced chemiluminescence kit.

Measurement of Src kinase activity. Src kinase was measured by a radioenzyme assay using a Src kinase assay kit (6). The assay system was based on phosphorylation of a specific substrate peptide [p34cdc2-(6–20)] by the transfer of [γ-32P]ATP by Src kinase. Aliquots (1.0 ml each) of the smooth muscle cell suspension (2 × 10^6 cells/ml) were incubated with reagents from 15 s to 20 min at 37°C. The incubation was stopped with 1 ml of chilled HEPES buffer. The suspensions were immediately centrifuged at 10,000 rpm for 50 s at 4°C in a microfuge, and the supernatant was removed. The resultant pellet was resuspended in 30 µl of chilled HEPES buffer (pH 7.4) containing 50 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 20 mM EDTA, 15 mM MgCl2, 1 mM DTT, 25 µg/ml leupeptin, and 25 µg/ml aprotinin (extraction buffer). Each suspension (20–30 samples) was immediately frozen in liquid nitrogen and was stored at −70°C overnight. The suspensions were thawed and sonicated for 30 s at 4°C. The sonicates were vortexed, allowed to settle for 10 min at 4°C, and then centrifuged at 10,000 rpm for 15 min at 4°C. Each 10-µl sample of the supernatant (−30 µg protein) was combined with 20 µl of substrate solution, which contained 10 ng µg Src substrate peptide [p34(cdc2 (6–20)] Lys-Val-Glu-Lys-Ile-Glu-Gly-Thr-Tyr-Gly-Val-Tyr-Lys), 50 mM Tris-HCl, 62.5 mM MgCl2, 12.5 mM MnCl2, 1 mM EDTA, 0.125 mM sodium orthovanadate, and 1 mM DTT (assay dilution buffer, pH 7.2). For the control reactions, 10 µl of the supernatant from corresponding cell extracts were combined with 20 µl of assay dilution buffer without Src substrate peptide. All procedures were performed at 37°C. Aliquots (10 µl) of [γ-32P]ATP (2 µCi) dissolved in 2× assay dilution buffer, 500 µM ATP, and 75 mM MnCl2 were added to each sample at 30°C. After incubation for 10 min at 30°C, the reaction was stopped with 20 µl of ice-cold 40% TCA. All samples were placed on ice for 10 min and were centrifuged at 10,000 rpm for 10 min to precipitate any extract proteins. Supernatant (25 µl from each tube; total volume, 60 µl) was removed, spotted on separate p81 phosphocellulose papers, and mounted on a piece of aluminum foil. Each disk was placed in a glass scintillation vial containing 15 ml of 0.75% phosphoric acid. After 30 min of mixing at room temperature, the washing reagent was decanted. Each paper was mixed with 10 ml of 0.75% phosphoric acid, followed by 10 ml acetone, for 10 min at room temperature. Each reagent was decanted after each washing. Finally, 10 ml of scintillant were added, and the radioactivity remaining on each binding paper was counted in a liquid scintillation counter. Nonspecific binding of [γ-32P]ATP to the binding paper without substrate was subtracted from each control sample that included the substrate. Src kinase activities were expressed as picomoles per minute per milligram protein. The protein in each sample (same fractions as Src kinase measurements) was measured using a Bio-Rad protein assay system.

Measurement of contraction. Aliquots consisting of 2.5 × 10^4 cells in 0.5 ml of medium were added to 0.1 ml of a solution containing the test agents. The agents were agonists or combinations of agonists and inhibitors. The reaction was interrupted by the addition of 0.1 ml of acrolein at a final concentration of 1% (vol/vol). Individual cell length was measured by computerized image micrometry. The average length of cells in the control state or after addition of test agents was obtained from 50 cells encountered randomly in successive microscopic fields. The contractile response is defined as the decrease in the average length of the 50 cells and is expressed as the absolute change or the percent change from control length (1).

Preparation of permeable smooth muscle cells and preincubation with Gβγ antibody. In experiments involving preincubation of smooth muscle cells with Gβγ antibody, muscle cells were made permeable without affecting their overall function (1). The partially digested muscle tissue was washed with 50 ml of a “cytotoxic” enzyme-free medium (cytotoxic buffer) of the following composition (in mM): 20 NaCl, 100 KCl, 5.0 MgSO4, 0.96 NaH2PO4, 25.0 NaHCO3, 1.0 EGTA, and 0.48 CaCl2. The medium contained 2% BSA and was equilibrated with 95% O2-5% CO2 to maintain a pH of 7.2. Muscle cells were allowed to disperse spontaneously in this medium and were harvested by filtration on 500 µm Nitex mesh. Isolated cells were permeabilized by incubation for 3 min in saponin (75 µg/ml). The cell suspension was centrifuged down and was resuspended in the cytotoxic buffer containing antitymcin A (10 µM), ATP (1.5 mM), and an ATP-regenerating system consisting of creatine phosphate (5 µM) and creatine phosphokinase (10 U/ml). The permeabilized cells were allowed to rest in a 95% O2-5% CO2 environment for 30 min. Five million cells in 0.1 ml cytotoxic buffer were incubated with 2 µl of Gβγ antibody for 1 h before the addition of agonists.

RESULTS

C2 Ceramide and Endothelin Induced PI 3-kinase Activities, and the Activation was Ca2+ Dependent

Our previous studies have shown that both C2 ceramide and endothelin induced contraction of smooth muscle cells at 30 s and 4 min (2, 6, 16). Their effect on PI 3-kinase activity was studied in this paper. C2 ceramide, the permeable short-chain C2 ceramide (10−7 M), increased PI 3-kinase activity at 30 s and 4 min (154.3 ± 3.2 and 198.6 ± 4.2%, respectively, compared with control, P < 0.05). In cells incubated in 0 Ca2+ (2 mM EGTA) there seems to be no significant difference (P = 0.25) in activity increments at 30 s and 4 min (103.6 ± 2.8 and 122.7 ± 3.8%; Fig. 1A). A similar result was found with endothelin; kinase activities increased at 30 s and 4 min (151.5 ± 8.5 and 175.8 ± 8.7%) but were much less in 0 Ca2+ (119.5 ± 7.8; Fig. 1B). These results show that both C2 ceramide and endothelin stimulated PI 3-kinase activity at 30 s and 4 min and to a much lesser extent in 0 Ca2+−2 mM EGTA. These data were corroborated on Western immunoblotting (Fig. 2, A and B) by using monoclonal PI 3-kinase p85 antibody.

C2 Ceramide and Endothelin Induce PI 3-Kinase Activities Via Different Pathways

We next investigated whether C2 ceramide and endothelin activated the same signal transduction pathway in the activation of PI 3-kinase. We have previously shown (6) that C2 ceramide-induced Src kinase activi-
ties are through a cascade whereby PI 3-kinase is downstream of Src kinase in the signal transduction cascade involving the protein tyrosine kinases (see model in Fig. 3). Herbinycin A (3 µM), an Src kinase inhibitor, was used in our study to see if it has any effect on agonist-induced PI 3-kinase activities.

There was a significant inhibition of C₂ ceramide-induced PI 3-kinase activities by herbinycin A at 30 s (101.3 ± 11.0%, P < 0.05, n = 4) and at 4 min (63.8 ± 6.8%, P < 0.05, n = 4; Fig. 4). The inhibitory effect of genistein (3 µM), a tyrosine kinase inhibitor, and herbinycin A (3 µM) was tested on Western immunoblotting, showing lower-density bands in herbinycin A- and genistein-pretreated cells than C₂ ceramide alone (Fig. 2A). Thus the data suggest that C₂ ceramide-induced activation of PI 3-kinase was through an Src kinase-related protein tyrosine kinase cascade and that PI 3-kinase was downstream of Src kinase.

There was a slight inhibition of endothelin-induced PI 3-kinase activities by herbinycin A at 30 s (129.2 ± 4.7%, P < 0.05, n = 4) and at 4 min (144.2 ± 13.2%, P < 0.05, n = 4; Fig. 5). Western immunoblotting supported these results (Fig. 2B). The data suggest that endothelin-induced activation of PI 3-kinase was not dependent on Src kinase and that a tyrosine kinase was upstream of PI 3-kinase (see model in Fig. 3). Western immunoblotting showed that preincubation with genistein inhibited endothelin-induced PI 3-kinase activation (Fig. 2B).

The same results relative to PI 3-kinase activation by C₂ ceramide and endothelin were found on Western immunoblotting using immunoprecipitates of PI 3-kinase subunit p85 followed by immunoblotting with phosphorylated tyrosine kinase antibody (Fig. 2C).

Another experiment was done using Western immunoblotting (Fig. 6C) to find out if C₂ ceramide or endothelin phosphorylated Src kinase in the protein tyrosine kinase-PI 3-kinase cascade. C₂ ceramide, not endothelin, phosphorylated Src kinase; an increase in the protein phosphorylation corresponding to Src kinase (60 kDa) was notable in response to C₂ ceramide but not endothelin. Src kinase activity assay showed that there is a significant increase of Src kinase activity (units: pmol·min⁻¹·mg protein⁻¹) stimulated by C₂ ceramide at 30 s and 4 min (3.47 and 4.13) compared with control (1.27), whereas endothelin showed almost no activation of Src kinase at 30 s and 4 min (1.26 and 1.27). Thus results from Western blotting of phosphorylated Src kinase and from measurement of Src kinase activity confirmed that endothelin-induced activation of PI 3-kinase is Src kinase independent.

C₂ Ceramide and Endothelin Activate Different Types of PI 3-Kinase

We investigated whether C₂ ceramide or endothelin interacted with different catalytic subunits. At least two groups of PI 3-kinase have been identified (18), namely, type IA with p85 as regulatory site and p110α or p110β as catalytic sites and type IB with uncertain regulatory site and p110γ as catalytic site. Three antibodies (either anti-p85, anti-p110α, or anti-p110γ) were used in Western immunoblotting (Figs. 2 and 6) to detect the proteins; higher-intensity bands relative to control were observed when endothelin-treated cells were probed with these three separate antibodies, whereas, in C₂ ceramide-treated samples, higher-intensity bands than control were seen only when the cells were probed with anti-p85 and anti-p110α but not with anti-p110γ antibodies. To assess if other tyrosine-containing proteins bind to PI 3-kinase or Src kinase and whether they can also be phosphorylated when stimulated by C₂ ceramide and endothelin, we immu-
nprecipitated with either the PI 3-kinase subunit antibody or Src kinase antibody. A number of unknown proteins were found to bind to PI 3-kinase and Src kinase when Ponceau S concentrate, a protein-staining reagent, was applied to view the bands (data not shown). After immunoprecipitation was performed using P-Tyr (PY99) phosphotyrosine antibody, only PI 3-kinase subunits and Src kinase appeared on the blots. Immunoprecipitation with anti-p85, anti-p110α, or anti-p110γ in PI 3-kinase activity assay (Fig. 7) showed that endothelin interacted with all of these subunits (darker bands than control), suggesting that both type IA and type IB PI 3-kinase were activated by endothelin. C2 ceramide only interacted with p85 and p110α but not with p110γ subunits, indicating that only type IA PI 3-kinase was involved in C2 ceramide-induced activation. In summary, C2 ceramide interacted with type IA PI 3-kinase, and endothelin interacted with both type IA and type IB PI 3-kinase.

Endothelin-Induced PI 3-Kinase Activation Is Through G Protein βγ-Subunit

We have previously shown (2) that specific G proteins mediated endothelin-induced contraction. It was recently reported that a catalytic subunit of PI 3-kinase (p110γ) was greatly stimulated by Gβγ (11). Pretreatment of the cells with a specific monoclonal antibody to the βγ-subunit of G protein was used in saponified smooth muscle cells to block the G protein βγ-subunit. Figure 8 shows no changes in saponified cells in the control state compared with unsaponified cells. When the G protein βγ-subunit was blocked, C2 ceramide, not endothelin, activated PI 3-kinase catalytic subunit p110α. In addition, endothelin and C2 ceramide did not activate PI 3-kinase when p110γ was used to immunoprecipitate PI 3-kinase (data not shown). These results confirmed that C2 ceramide activated only the p110α subunit and that endothelin activated PI 3-kinase through a G protein βγ-subunit.

Smooth Muscle Contraction Induced by C2 Ceramide and Endothelin Responded Differently to the Src Kinase Inhibitor Herbimycin A

Our previous study found that both C2 ceramide and endothelin induced contraction in colonic smooth muscle cells (1, 6). In this study, cells were preincubated with either the tyrosine kinase inhibitor genistein (3 μM) or herbimycin A (3 μM) for 20 min before the addition of either C2 ceramide (10−7 M) or endothelin (10−7 M). The result showed that there is a
Fig. 3. Direct activation of Src kinase pathway by C2 ceramide results in interaction of only type IA. Endothelin activates type IA and IB PI 3-kinase by interacting with the p85 regulatory subunit first, which in turn binds to the NH2 terminus of the catalytic subunits. Activation of both type IA and IB by endothelin is mediated by βγ-subunits of G proteins. TK, tyrosine kinase; Smase, sphingomyelinase.

Fig. 4. Percent increase of C2 ceramide (10^{-7} M)-induced PI 3-kinase activities upon preincubation of smooth muscle cells with herbimycin A (3 µM). Data are means ± SE from 5 separate experiments; P < 0.05 by one-way ANOVA.

Fig. 5. Percent increase of endothelin (10^{-7} M)-induced PI 3-kinase activities upon preincubation of smooth muscle cells with herbimycin A (3 µM). Data are means ± SE from 5 separate experiments; P < 0.05 by one-way ANOVA.
total inhibition of C2 ceramide-induced contraction by both inhibitors. Percent decrease in cell length was as follows: 39.05 ± 2.96% vs. 4.6 ± 1.12% in the cells preincubated with genistein and 3.08 ± 0.3% in the cells preincubated with herbimycin A. Endothelin-induced contraction was inhibited by preincubation with genistein as follows: percent decrease in cell length was 44.3 ± 0.54% vs. 5.54 ± 2.0% but no significant inhibition by preincubation with herbimycin A (36.34 ± 0.76%) occurred. These results confirmed that C2 ceramide-induced smooth muscle contraction was by the activation of a Src kinase cascade.

**DISCUSSION**

We have investigated the different signal transduction mechanisms of contraction induced by endothelin and C2 ceramide, specifically in their respective activation of PI 3-kinase. Both C2 ceramide and endothelin induce contraction of smooth muscle cells isolated from the gastrointestinal tract. PI 3-kinase is activated by various stimuli, including growth factors, hormones, and cytokines. The activation of PI 3-kinase leads to the phosphorylation of a catalytic subunit, p110α, which is critical for the downstream signaling events that mediate the contraction response. Our results show that endothelin activates PI 3-kinase by phosphorylating the p110α catalytic subunit, whereas C2 ceramide does not. This difference in activation could be due to the distinct mechanisms by which endothelin and C2 ceramide induce contraction. Endothelin activates PI 3-kinase through a complex signaling cascade involving the activation of Src kinase, which phosphorylates the p110α catalytic subunit. In contrast, C2 ceramide activates PI 3-kinase through a different pathway, possibly involving the activation of a different Src kinase or a different Src kinase cascade.

Fig. 6. A: top, result of IP using a phosphotyrosine monoclonal antibody followed by Western IB using a PI 3-kinase monoclonal antibody specific for PI 3-kinase p110α subunit. Cells were stimulated by either C2 ceramide or endothelin in 2 mM CaCl2 HEPES buffer. Both C2 ceramide (10^{-7} M) and endothelin (10^{-7} M) seemed to phosphorylate the p110α catalytic subunit of PI 3-kinase; bottom, result of IP using a PI 3-kinase monoclonal antibody specific for PI 3-kinase p110α subunit followed by Western IB using a phosphotyrosine monoclonal antibody. Cells were stimulated by either C2 ceramide or endothelin in 2 mM CaCl2 HEPES buffer. Both C2 ceramide (10^{-7} M) and endothelin (10^{-7} M) seemed to phosphorylate the p110α catalytic subunit of PI 3-kinase.

B: top, result of IP using a phosphotyrosine monoclonal antibody followed by Western IB using a PI 3-kinase monoclonal antibody specific for PI 3-kinase p110α subunit. Cells were stimulated by either C2 ceramide or endothelin in 2 mM CaCl2 HEPES buffer. Endothelin, but not C2 ceramide, seemed to phosphorylate p110α catalytic subunit of PI 3-kinase; bottom, result of IP using a PI 3-kinase monoclonal antibody specific for PI 3-kinase p110α subunit followed by Western IB using a phosphotyrosine monoclonal antibody. Cells were stimulated by either C2 ceramide or endothelin in 2 mM CaCl2 HEPES buffer. Endothelin (10^{-7} M), but not C2 ceramide (10^{-7} M), seemed to phosphorylate p110α catalytic subunit of PI 3-kinase.

C: top, result of IP using a phosphotyrosine monoclonal antibody followed by Western IB using a pp60src kinase monoclonal antibody. Cells were stimulated by either C2 ceramide or endothelin in 2 mM CaCl2 HEPES buffer. C2 ceramide, but not endothelin, seemed to phosphorylate pp60src kinase; bottom, result of IP using a pp60src kinase monoclonal antibody followed by Western IB using a phosphotyrosine monoclonal antibody. Cells were stimulated by either C2 ceramide or endothelin in 2 mM CaCl2 HEPES buffer. C2 ceramide, but not endothelin, seemed to phosphorylate pp60src kinase.


Fig. 8. Results of IP of cells preincubated with the specific monoclonal antibody to βγ-subunit of G protein. IP was done using a PI 3-kinase monoclonal antibody specific to PI 3-kinase p110α subunit followed by Western IB using a phosphotyrosine monoclonal antibody. No changes were observed in saponified cells in control state compared with unsaponified cells. Upon preincubation of the cells with G protein βγ-subunit antibody, C2 ceramide, not endothelin, activated PI 3-kinase catalytic subunit p110α.
the rabbit colon (1, 6). Endothelin is a potent vasoconstrictor. Its physiological importance in the control of gastrointestinal motor function has been investigated (2). Endothelin receptors are present on smooth muscle cells in jejunum, ileum, and colon (7, 23). C2 ceramide is the product of hydrolysis of sphingomyelin and acts as an intracellular messenger that mediates sustained smooth muscle contraction (16).

We have previously shown on Western immunoblotting that C2 ceramide-induced contraction is mediated by an increase in PI 3-kinase (32). We have further shown that, in C2 ceramide-induced contraction, PI 3-kinase is downstream of Src kinase in the protein tyrosine kinase cascade. Our data indicate that C2 ceramide increased PI 3-kinase activities at both 30 s and 4 min after stimulation with C2 ceramide. This increase in PI 3-kinase activities was inhibited by preincubation of the cells with genistein (a tyrosine kinase inhibitor) or preincubation with herbimycin A (an Src kinase inhibitor; Figs. 2A and 4). There was a close correlation between the Ca2+ requirement for C2 ceramide-induced contraction and C2 ceramide-induced PI 3-kinase activity. The data indicate that the sustained PI 3-kinase activity in response to C2 ceramide (4 min) is dependent on Ca2+ from extracellular sources (Fig. 1). Extracellular Ca2+ is also required to maintain sustained contraction induced by C2 ceramide at 4 min (6).

Similar results as above were found in response to stimulation by endothelin. It has been reported that, in vascular smooth muscle cells, endothelin acts either to mobilize intracellular Ca2+ or to promote Ca2+ influx (13, 33). Our results indicate that, in smooth muscle cells from the rabbit colon incubated in buffer lacking extracellular Ca2+ (0 Ca2+-2 mM EGTA), endothelin-induced sustained contraction is inhibited at 4 min (2). The contractile response to endothelin seems to be in accord with endothelin-induced PI 3-kinase activity. PI 3-kinase activity in response to endothelin is greatly inhibited at 4 min stimulation in the presence of 0 Ca2+ -2 mM EGTA (Figs. 1B and 2B).

Endothelin seems to activate PI 3-kinase in a G protein-mediated pathway, independent of the Src kinase pathway. The data indicate that, on Western immunoblotting (Fig. 6C), endothelin-induced contraction was not accompanied by any Src kinase phosphorylation. Furthermore, endothelin did not induce any increase in Src kinase activity in association with an increase in PI 3-kinase activation (Fig. 7). Endothelin-induced PI 3-kinase activation is through the G protein βγ-subunit, which corresponds to the finding on Western blotting (Fig. 8), whereby preincubation with the G protein βγ-subunit antibody blocked the endothelin-induced PI 3-kinase activation. We have previously shown (2) that specific G proteins mediated endothelin-induced contraction. The transmembrane signaling of endothelin is through two specific GTP-binding components that are Gαi; a G13-like protein appeared to be required for the initial rapid transient contraction, and a G11-like protein is required for the sustained phase of the contraction. It was recently reported that the catalytic subunit of PI 3-kinase (p110γ) was greatly stimulated by Gβγ (11). It was also suggested that Gβγ can bind directly to p110β (10). Thus it is possible for an agonist like endothelin, which acts through a G protein-mediated response (2), to activate Gβγ and thus interact with either or both p110β and p110γ catalytic subunits. Therefore, endothelin most likely activates the PI 3-kinase catalytic subunit independent of Src kinase.

It was recently found that serine kinase is tightly associated with PI 3-kinase and acts as a negative regulator of this enzyme; the best substrate found is the p85 subunit of PI 3-kinase (4). When PI 3-kinase p85 subunit immunoprecipitates were immunoblotted using serine phosphorylated antibody, no phosphorylated serine signals at the position of 85 kDa were found in response to the stimulation by C2 ceramide and endothelin (data not shown). Therefore, serine kinase did not seem to be involved in the activation of PI 3-kinase induced by C2 ceramide and endothelin in rabbit colonic smooth muscle cells.

Type IA PI 3-kinase enzymes purify as heterodimers with a molecular mass of ~200 kDa containing a catalytic subunit of 110 kDa (p110) and a regulatory subunit p85 (10, 25, 26). Several mechanisms for regulating their enzymatic activities in response to extracellular stimuli have been elucidated. Type IA members p110α, -β, and -δ are stimulated by tyrosine phosphorylated proteins through interaction with regulatory PI 3-kinase subunits such as p85 or p55 (5, 30). They in turn bind to the NH2 terminus of the catalytic p110 subunit, thereby inducing PI 3-kinase activity. In contrast, type IB members have the catalytic subunit p110 (21). Our results indicate that C2 ceramide interacted only with type IA PI 3-kinase and through the tyrosine kinase cascade pathway, whereas endothelin interacted with both IA and IB types of PI 3-kinase. The involvement of type IB PI 3-kinase in endothelin activation fits the hypothesis that endothelin interacts with G protein in the cell membrane, activates PI 3-kinase without activating Src kinase, and induces smooth muscle contraction. Type IA was reported to be regulated by tyrosine kinase and Ras (29). We hypothesize that smooth muscle cells of the rabbit colon have different types of PI 3-kinase that could be activated differentially and specifically by different contractile agonists. Our data suggest that a direct activation of the Src kinase pathway results in the interaction with only type IA, whereas endothelin activates type IA and IB PI 3-kinase, probably through activation of a tyrosine kinase first followed by the binding of the p85 regulatory subunit to the NH2 terminus of the p110α or p110γ catalytic subunit. Activation of both classes IA and IB by endothelin is mediated by G proteins.
REFERENCES


