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Activation of phospholipase D in porcine tracheal smooth muscle: role of phosphatidylinositol 3-kinase and RhoA activation

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Abstract

Muscarinic receptor agonists transiently activate phospholipase D in tracheal smooth muscle. Muscarinic activation of phospholipase D in this tissue is dependent on activation of protein kinase C and an unidentified pathway that is not protein kinase C dependent. Cholinergic agents have also been shown to activate phospholipase D by pathways linked to the small G protein, RhoA. This study explores the relationship between muscarinic activation of phophatidylinositol 3-kinase and activation of RhoA, and examines whether phospholipase D activation is dependent on either pathway in tracheal smooth muscle. Wortmannin or 2-(4-morphonyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY-294002), putative specific inhibitors of phophatidylinositol 3-kinase, significantly inhibit acetylcholine-induced formation of phosphatidylethanol and also block acetylcholine-induced translocation of RhoA to the membrane. In previous experiments calphostin C, a protein kinase C inhibitor, partially inhibited both acetylcholine-induced and phorbol-12-myristate-13-acetate (PMA)-induced phosphatidylethanol formation. In the present study calphostin C did not block acetylcholine-induced RhoA translocation to the membrane. However, the Rho kinase inhibitor, *N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632), significantly inhibited acetylcholine-induced phosphatidylethanol formation, but had no effect on activation of phospholipase D by PMA. Acetylcholine treatment also stimulated the phosphorylation of the 110-kDa subunit of phosphatidylinositol 3-kinase. Phosphorylation of phosphatidylinositol 3-kinase 110-kDa subunit could be blocked by wortmannin in a concentration-dependent manner, and acetylcholine-induced phosphatidylinositol 3-kinase activity was significantly inhibited by wortmannin. LY-294002 also inhibited acetylcholine-induced phosphorylation of 110-kDa subunit and activation of phosphatidylinositol 3-kinase. These results suggest that acetylcholine stimulation translocates RhoA to the membrane by a phosphatidylinositol 3-kinase-dependent mechanism and acetylcholine-induced phospholipase D stimulation is at least partly mediated via phosphatidylinositol 3-kinase, however, protein kinase C appears to activate phospholipase D independent of phosphatidylinositol 3-kinase or RhoA activation in porcine tracheal smooth muscle. © 2001 Published by Elsevier Science B.V.

Keywords: Phospholipase D; Phosphatidylinositol 3-kinase; Acetylcholine; Airway smooth muscle; RhoA

1. Introduction

We have previously demonstrated that phospholipase D in intact tracheal smooth muscle strips can be significantly, albeit transiently, activated by acetylcholine receptor agonists (Mamoon et al., 1999a,b). Acetylcholine-induced phospholipase D activation in this tissue appears to be mediated via both protein kinase C-dependent and protein kinase C-independent pathways (Mamoon et al., 1999a). Several lines of evidence suggest that Rho proteins (small G protein, GTPases) can also control signaling to phospholipase D (Schmidt et al., 1999; Vinggaard et al., 1997). Translocation of cytosolic RhoA to the plasma membrane has been demonstrated in human embryonic kidney cells after muscarinic acetylcholine receptor activation (Keller et al., 1997). It has been shown that RhoA is necessary for the activation of phospholipase D in human promyelocytic leukemic cells (Abousalham et al., 1997) and rat adipocytes (Karnam et al., 1997). It has also been demonstrated that Clostridium difficile toxin B which glycosylates Rho protein can efficiently block muscarinic acetylcholine receptor-stimulated phospholipase D activity in human embryonic kidney cells (Keller et al., 1997; Schmidt et al., 1996). Furthermore, Schmidt et al. (1999) have demonstrated that

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RhoA-dependent phospholipase D activation in human embryonic kidney cells is also dependent on RhoA-stimulated serine/threonine kinase, Rho-kinase.

Alternative pathways for activation of phospholipase D have been suggested. There are indications that the enzyme may be activated, at least partially, via phosphatidylinositol 3-kinase pathway (Cissel et al., 1998; Kozawa et al., 1997). This has also been demonstrated in neutrophils (Nakamura et al., 1997) and rat adipocytes where phosphatidylinositol 3-kinase activity was blocked by wortmannin (Standaert et al., 1996), a potent inhibitor of the phosphatidylinositol 3-kinase family of enzymes (Nakanishi et al., 1992; Powis et al., 1994; Tomiyama et al., 1995). Phosphatidylinositol 3-kinase, ubiquitous in mammalian cells, is a major enzyme involving lipid second messengers (Toker and Cantley, 1997). It consists of a 110-kDa (α, β, γ or δ) catalytic subunit and an 85-kDa regulatory subunit. However, the functions of the different subtypes have not been well characterized (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997). Activation of phosphatidylinositol 3-kinase results in phosphorylation of phosphatidylinositide at the D-3 position of the inositol ring resulting in an increase in intracellular levels of D-3 phosphorylated phosphatidylinositides such as phosphatidylinositide (3)P (PIP), phosphatidylinositide (3,4)P_2 (PIP_2) and phosphatidylinositide (3,4,5)P_3 (PIP_3). These lipid products of phosphatidylinositol 3-kinase activation have been demonstrated to be second messengers in their own right (Stephens et al., 1993; Toker and Cantley, 1997).

Mammalian cells express at least two isoforms of phospholipase D, phospholipase D_1 and phospholipase D_2 (Exton, 1999). One of the dominant products of phosphatidylinositol 3-kinase activation, PIP_2, has been shown to be an essential cofactor for phospholipase D activation (Frohman et al., 1999). It has also been reported that another product of phosphatidylinositol 3-kinase activation, PIP_3, can also activate the enzymes with equal potency but reduced efficacy (Frohman et al., 1999). The major product of phospholipase D activation is phosphatidic acid. Phosphatidic acid, subsequent to phospholipase D activation, is thought to serve distinct signaling functions including activation of cellular kinases and phospholipases as well as low molecular-weight G proteins (English, 1996). Phosphatidic acid is also considered to behave as a reservoir for diacylglycerol (an activator of protein kinase C) since phosphatidic acid can both be converted to or generated from diacylglycerol (Dhalla et al., 1997). Therefore, it is important to understand how phospholipase D is stimulated in mammalian cells. Based on the above information and also in context to our previous findings that part of acetylcholine-induced phospholipase D activation in tracheal smooth muscle is not protein kinase C-dependent, we have examined the possible role of phosphatidylinositol 3-kinase in muscarinic-agonist induced phospholipase D activation and RhoA activation in intact tracheal smooth muscle.

2. Materials and methods

2.1. Materials

[^{32}P]-Orthophosphate, [^{32}P]-ATP and [9,10-^{3}H(N)] palmitic acid were obtained from DuPont-New England Nuclear (Boston, MA). Antibodies to RhoA and phosphatidylinositol 3-kinase p110, protein A/G PLUS-Agarose and molecular weight markers were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acetylcholine chloride, wortmannin, 2-(4-morphonyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY-294002) and phorbol-12-myristate-13-acetate (PMA) were from Sigma (St. Louis, MO). Phosphatidic acid and phosphatidylethanol standards were from Avanti Polar Lipids (Alabaster, AL). Super-Signal west dura extended duration substrate (Luminol) and Micro Bicinchoninic acid Protein Assay Kit were from Pierce Chemical (Rockford, IL). *N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) was a gift from Yoshitomi Pharmaceutical Industries (Japan). All materials for gel preparations were obtained from either Sigma or Bio-Rad Laboratories (Hercules, CA).

2.2. Muscle strip preparation

Male Yorkshire pigs (weight, 30–35 kg) were anesthetized with 5% isoflurane in 2 l/min O_2 and sacrificed by exsanguination. The trachea was quickly removed, irrigated with 0.9% NaCl, and transferred to a sterile container containing normal external solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5.5 mM glucose, 0.5 ml penicillin–streptomycin and 10 mM HEPES buffer, pH adjusted to 7.4). The trachea was cut open longitudinally, and smooth muscle was dissected free of epithelium, gland cells, connective tissue and cartilage. The smooth muscle was then carefully cut into single ring-wide segments. Muscle strips were maintained at 37 °C in an atmosphere containing 5% CO_2 in Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 12 mM glucose, pH adjusted to 7.4).

2.3. Assay for phospholipase D activation

2.3.1. Radiolabeling

Phospholipids were labeled by incubating tracheal smooth muscle segments (1 ring) for 6 h at 37 °C in 3 ml Krebs–Henseleit buffer with 3 μCi/ml [^{3}H]palmitic acid. At the end of the incubation period, tissue was washed three times with Krebs–Henseleit buffer and maintained in 1 ml of the same buffer. Acetylcholine and Y-27632 were diluted with Krebs–Henseleit buffer. Calphostin C, LY-294002 and wortmannin were dissolved in dimethyl sulfoxide (DMSO) and then diluted with buffer to final working concentrations. Control strips were treated with equal

amounts of DMSO (0.01–0.1%). Ethanol (100 mM) was added 10 min before the addition of the pharmacological agents or vehicle.

2.3.2. Lipid extraction

Lipids were extracted by a modified Bligh and Dyer (1959) procedure. Briefly, incubations were stopped by addition of 4 ml of methanol containing 2% acetic acid. Methylene chloride (2 ml) was added, and the sample was homogenized and shaken vigorously. Samples were allowed to stand at room temperature for 30–60 min. Another 2 ml of methylene chloride and 2 ml of 1 M KCl were then added. The organic and aqueous phases were separated by centrifugation after which the organic phase was collected and evaporated under a stream of nitrogen. The extracted material was then dissolved in 100 μl of methylene chloride/methanol (1:1) and stored at −20 °C.

2.3.3. Thin layer chromatography

Phosphatidylethanol was separated using one-dimensional thin layer chromatography (TLC) plates. Aliquots of 20 μl were spotted on the TLC plates; the plates were then developed using the upper phase of a solvent system consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10). Radioactive phospholipids were located on the plate using a beta emission scanner (Bioscan, Washington, DC, USA) and by comparing the position of radioactive material to the position of phosphatidic acid or phosphatidylethanol standards, which were run on the same plate. Areas corresponding to phosphatidylethanol standards were scraped from the plates, and the radioactivity measured by scintillation spectroscopy.

2.4. Identification and agonist-induced phosphorylation assay of phosphatidylinositol 3-kinase p110

Muscle strips were cut into small pieces, homogenized in 3 ml modified radioimmunoprecipitation (RIPA) buffer with protease inhibitors (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mg/ml phenylmethylsulfonylfluoride, 30 μl/ml aprotinin and 100 mM sodium orthovanadate, pH adjusted to 7.4) using a homogenizer, and then incubated on ice for 30 min. Cellular debris was pelleted by centrifugation at 900 × *g* at 4 °C for 15 min. One milliliter of supernatants containing total cell lysates were collected in microcentrifuge tubes. Primary antibodies to phosphatidylinositol 3-kinase p110 (2 μg) were then added to each tube and incubated for 1 h at 4 °C. Protein A/G PLUS-Agarose (20 μl) were then added to each sample and rocked at 4 °C for 1 h. Immunoprecipitates were collected by centrifugation at 3000 rpm for 5 min and pellets were washed four times with phosphate buffered saline. After final wash supernatants were discarded, 40 μl of electrophoresis sample buffer added to each sample and boiled for 90 s. Proteins were separated on a 10% SDS gel and transferred to nitrocellulose membrane. The membranes were treated with horseradish peroxidase conjugated secondary antibody (anti-rabbit Ig), diluted to 1:20,000. Non-specific binding was blocked with 5% dried milk in Tris buffered saline, pH 8.0 with 0.05% Tween-20. Protein bands were visualized using an enhanced chemiluminescent detection kit (Luminol, Pierce).

For phosphorylation assays, muscle strips were pre labeled [^{32}P]-orthophosphate (1 mCi/ml) for 6 h. After stopping incubations with agonist or antagonists, cell lysates containing labeled proteins were immunoprecipitated with phosphatidylinositol 3-kinase p110 antibody and collected as described above. Immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gels. The gels were dried and phosphorylated proteins were quantified by placing the gel onto a storage phosphor screen for up to 6 h and then by scanning the exposed plate with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.5. Phosphatidylinositol 3-kinase activity determination

Muscle strips were incubated in 2 ml Krebs–Henseleit buffer and kept at 37 °C in a water bath for 1 h. Agonists or antagonists were added for appropriate time periods and then reactions were stopped by snap-freezing in dry ice/methanol. Tissues were homogenized in RIPA buffer by using a polytron device over ice. The samples were allowed to sit on ice for 45 min. Precipitates were collected and centrifuged at 900 × *g* and cell lysates collected as described above. The phosphatidylinositol 3-kinase activity assay was adopted, with little modification, from methods described by Su et al. (1999). Briefly, 1 ml of cell lysate from a treated strip was placed in a microcentrifuge tube. Antibody to phosphatidylinositol 3-kinase p110 (1.5 μl) was added and rocked for 90 min at 4 °C. Fifty microliters of protein A/G Plus was then added to each sample and rocked at 4 °C for 2 h. Beads were spun out and washed three times with TBS (10 mM Tris–HCl, pH 8; 150 mM NaCl). The beads were resuspended in 30 μl phosphatidylinositol 3-kinase buffer (25 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.5 mM EGTA). The reaction was begun by adding kinase containing beads (10 μl), phosphatidylinositol 3-kinase substrate (0.2 mg/ml phosphatidylinositol sonicated in phosphatidylinositol 3-kinase buffer), and 10 μl of 20 μM [^{32}P]-ATP in ATP buffer (0.4 M ATP, 0.1 M $MgCl_2$, 1 μCi/ml [^{32}P]-ATP) at 30 °C and was stopped by adding 100 μl 1 N HCl. Lipids were extracted and resolved on TLC plates using a solvent system consisting of chloroform/methanol/ammonium hydroxide/water (45:35:1.5:8.5). Standards were run on the same plates and identified by charring. Plates were then exposed to phosphor screen for 72 h and [^{32}P] measured using the PhosphorImager.

2.6. Assessment of RhoA translocation to the membrane fractions by Western/immunoblot

After incubations with agonists or antagonists reactions were stopped by snap-freezing. As described above, tissues were then homogenized in ice-cold RIPA buffer with inhibitors. Samples were spun at $900 \times g$ to get rid of the cell debris and cell lysates were collected. To separate membrane and cytosolic fractions, samples were then centrifuged at $100{,}000 \times g$ at 4 °C for 60 min to obtain the cytosolic and membrane fractions. Pellets were resuspended and proteins were extracted by incubation for 30 min in homogenization buffer. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were then immunoblotted with antibody to RhoA (2 μg), treated with horseradish peroxidase secondary antibody and visualized by using a chemiluminescent detection kit (Pierce Chemical). The timing for incubations, the procedures for gel separation, and transfer to nitrocellulose membrane and detection of protein bands by chemiluminescent methods were the same as described above. Protein bands on the exposed films were then quantified by using a densitometer.

2.7. Protein assay

Protein levels were estimated by the method of Lowry (Lowry et al., 1951) using prepared reagents (Pierce Chemical) and BSA as the standard.

2.8. Data presentation

Data from three or more treatment groups or multiple treatment of the same group were compared using one-way or repeated-measures analysis of variance, respectively, followed by appropriate *t*-tests for multiple comparisons against a single control. All data are expressed as mean ± S.E.M. and are collected from at least three independent experiments using three different animals. Data were deemed significant when $p \leq 0.05$. All statistical analyses were performed using Primer of Biostatistics Software (Version 4).

3. Results

A unique property of phospholipase D, transphosphatidylation, is often used to confirm its activation. In this reaction, short-chain alcohols can replace water as a phospholipase D substrate and rather than producing a phosphatidic acid, a phosphoester of the alcohol is produced (Liscovitch, 1991). In our experiments, the tracheal smooth muscle strips were incubated with [^{3}H]palmitic acid to label the phospholipids and the levels of transphosphatidylation product, [^{3}H]phosphatidylethanol, were measured after various treatment conditions. Fig. 1. shows inhibition of

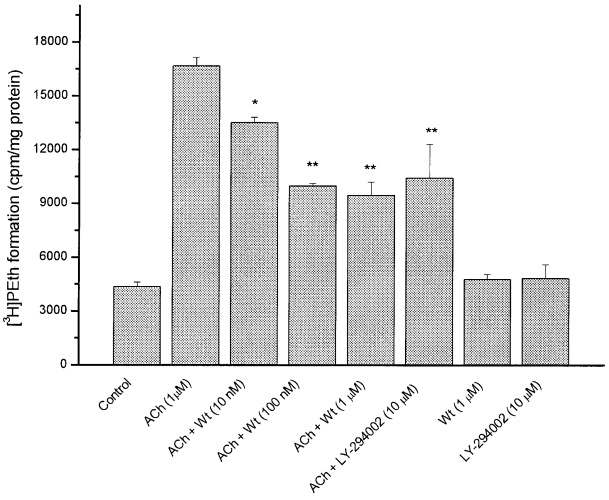

Fig. 1. Effect of wortmannin or Ly-294002 on acetylcholine-induced [^{3}H]phosphatidylethanol formation in porcine tracheal smooth muscle. Tracheal smooth muscle strips were labeled with [^{3}H]palmitic acid. Labeled strips were then pretreated with 100 mM ethanol for 10 min before exposing them to acetylcholine (1 μM) or vehicle. Wortmannin in increasing concentrations (10 nM–1 μM) or LY-294002 (10 μM) were added immediately prior to acetylcholine treatment. Incubations were stopped after 5 min. Values are presented as mean ± S.E.M. of three independent experiments. * $p = 0.0044$ and ** $p < 0.001$ compared with acetylcholine-treated group. ACh = acetylcholine, Wt = wortmannin and [^{3}H]Peth=[^{3}H]-phosphatidylethanol.

acetylcholine-induced phospholipase D activation by phosphatidylinositol 3-kinase inhibitors wortmannin and LY-294002. Stimulation of muscle strips by acetylcholine (1 μM) in the presence of ethanol for 5 min caused a significant increase (4368 ± 235 vs. 16685 ± 467 cpm/mg protein, control vs. acetylcholine, $p = 0.00019$) in [^{3}H]phosphatidylethanol formation. A similar 2–3 fold increase in [^{3}H]phosphatidylethanol formation was observed in previous experiments using 1 μM acetylcholine as an agonist (Mamoon et al., 1999a). All reactions were stopped at 5 min since in all previous experiments a peak level of [^{3}H]phosphatidylethanol formation was achieved around 5–6 min post-stimulation with acetylcholine. Compared to acetylcholine-treated group, [^{3}H]phosphatidylethanol levels were reduced by approximately 19% ($p = 0.00443$), 36% ($p = 0.00164$) and 43% ($p = 0.00121$) for concentrations of 10 nM, 100 nM and 1 μM wortmannin, respectively, added immediately prior to acetylcholine treatments. Wortmannin (1 μM) alone had no effect on phospholipase D basal activity (4368 ± 235 vs. 4789 ± 275 cpm/mg protein, control vs wortmannin, not significant). Another potent phosphatidylinositol 3-kinase inhibitor LY-294002, used at 10μM, also inhibited acetylcholine-induced phospholipase D activation by about 37% ($p = 0.00132$).

We have previously demonstrated that acetylcholine-induced phospholipase D activity is at least partly protein kinase C-dependent and PMA can activate phospholipase D in tracheal smooth muscle (Mamoon et al., 1999a). To

determine if phosphatidylinositol 3-kinase is involved in this pathway, the effect of wortmannin or LY-294002 on PMA-induced phospholipase D activation was studied. [^{3}H]phosphatidylethanol levels were measured after treatment of labeled muscle strips with 1 μM PMA (Fig. 2). Based on previous experiments, the tissues were exposed to 1 μM PMA for 5 min (Mamoon et al., 1999a). PMA increased [^{3}H]phosphatidylethanol levels by approximately 123% compared to the control group (9021 ± 265 vs. 3873 ± 363 cpm/mg protein, PMA vs. control, p = 0.00334). Wortmannin had no effect on PMA stimulated [^{3}H]phosphatidylethanol formation (9021 ± 265, PMA vs. 8439 ± 154, 10 nM wortmannin; 8436 ± 211, 100 nM wortmannin; 8738 ± 102, 1 μM wortmannin, not significant). LY-294002 (10 μM) also did not have any effect on PMA-induced phospholipase D activity (9021 ± 265 vs. 9086 ± 252 cpm/mg protein, PMA vs, LY-294002, not significant).

Treatment of intact tracheal smooth muscle strips with acetylcholine (1 μM) for 5 min resulted in translocation of cytosolic RhoA to membranes (19–26%, translocated from cytosolic to membrane fraction, not shown in Fig. 3). A densitometric measurement of Western blots of RhoA shows that in unstimulated tissue a small amount of RhoA is membrane-associated (Fig. 3). Compared to the unstimulated control group there was 6–8.5 fold increase in membrane-association of RhoA in acetylcholine-stimulated strips. The purpose of this experiment was also to examine the role of phosphatidylinositol 3-kinase or protein kinase C on acetylcholine-induced translocation of RhoA from the cytosol to the membrane. Wortmannin (1 μM), LY-294002 (10 μM) or the protein kinase C inhibitor, calphostin C (1 μM), were added to the incubation medium immediately prior to the addition of acetylcholine. The concentration of calphostin C was selected in the context of our earlier findings that it can inhibit phospholipase D activation by acetylcholine in intact tracheal smooth muscle (Mamoon et al., 1999a). After 5 min of incubation, the amount of RhoA in the wortmannin-treated membranes was decreased by 71 ± 5% as compared to the acetylcholine-induced levels of membrane-associated RhoA. LY-294002 also decreased membrane association by 63 ± 4%, however, calphostin C had no effect on acetylcholine-induced translocation of RhoA to the membrane fractions.

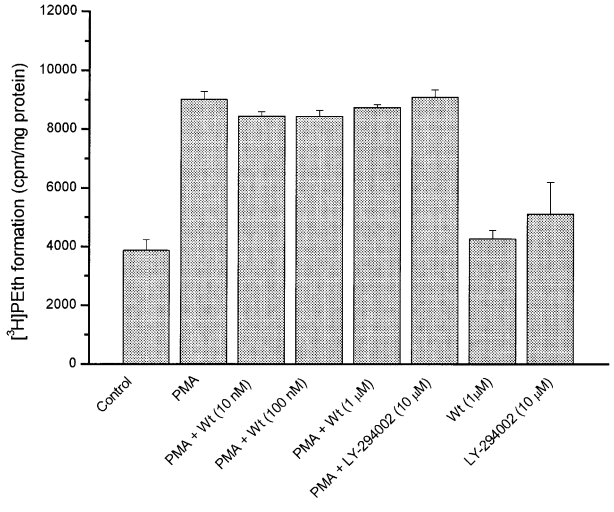

Fig. 2. Effect of wortmannin or LY-294002 on PMA-induced [^{3}H]phosphatidylethanol formation. Tracheal smooth muscle rings were labeled with [^{3}H]palmitic acid. Labeled muscle strips were then pretreated with 100 mM ethanol for 10 min before exposing them to PMA (1 μM) or vehicle. Wortmannin in increasing concentrations (10 nM–1 μM) and LY-294002 (10 μM) were added immediately prior to PMA treatment. Incubations were stopped after 5 min. Lipids were extracted and separated on one-dimensional TLC plates. Results are given as mean ± S.E.M. of three independent experiments. Wt = wortmannin and [^{3}H]Peth=[^{3}H]phosphatidylethanol.

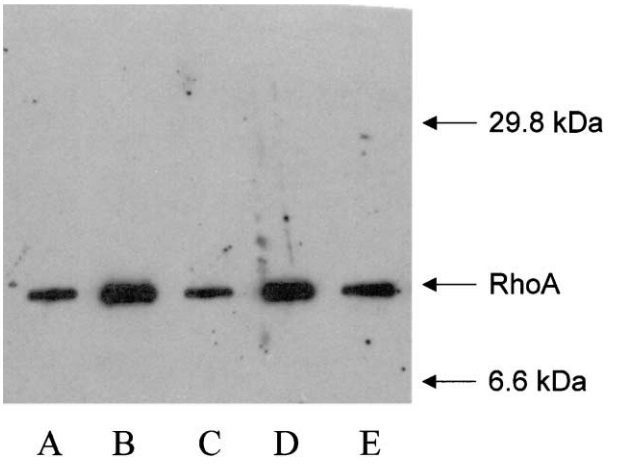

Fig. 3. Effect of various inhibitors on acetylcholine-induced translocation of RhoA to the membrane. A representative Western blot (one of three independent experiments) of RhoA in the membrane fractions is shown. All reactions were run for 5 min. Wortmannin (1 μM), calphostin C (1 μM) or LY-294002 (10 μM) were added immediately prior to acetylcholine (1 μM) treatment. Proteins in the membrane fractions were then extracted and separated on a 10% SDS-polyacrylamide gel. Proteins transferred to PVDF membrane were then blotted with anti-RhoA antibody (rabbit polyclonal, 1:300). Membranes were developed using an enhanced chemiluminescent detection kit. (A) no treatment; (B) acetylcholine; (C) acetylcholine + wortmannin; (D) acetylcholine + calphostin C; (E) acetylcholine + LY-294002.

Fig. 4 shows a representative autoradiogram of phosphatidylinositol 3-kinase-dependent phosphorylation of phosphatidylinositol resolved on a TLC plate. In our previous studies, we have shown that acetylcholine-induced phospholipase D activity was accomplished both via protein kinase C-dependent and protein kinase C-independent pathways (Mamoon et al., 1999a). However, it was not clear whether other intermediary steps were involved in the protein kinase C-independent pathway leading to muscarinic receptor agonist-induced activation of phospholipase D in tracheal smooth muscle. Therefore, we examined phosphatidylinositol 3-kinase activity as an alternative pathway activated by acetylcholine leading to phospholipase D activation. All incubations were stopped after 5 min. The relative phosphorylations of phosphatidylinositol from different strips were

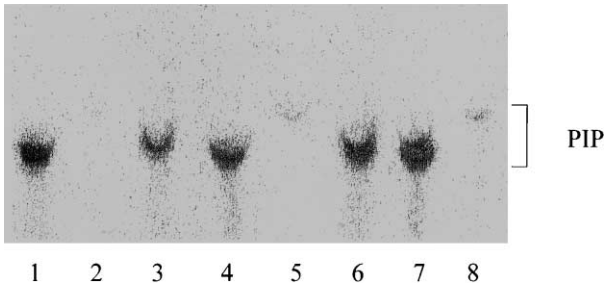

Fig. 4. Representative autoradiogram (one of three independent experiments) of phosphatidylinositol 3-kinase-dependent phosphorylation of TLC-purified phosphatidylinositol. Muscle strips were incubated with acetylcholine (1 μM) for 5 min. Where appropriate, wortmannin (10 nM–1 μM), LY-294002 (10 μM) or Y-27632 (1 μM, 10 μM) were added immediately prior to acetylcholine. As described in Section 2, cells lysates were incubated with primary antibody to phosphatidylinositol 3-kinase p110 (goat polyclonal, 1:500). Immunoprecipitated phosphatidylinositol 3-kinase p110 was then added to phosphatidylinositol 3-kinase buffer, ATP buffer containing [^{32}P]-ATP and phosphatidylinositol and incubated for 5 min at 30 °C. After stopping the reactions lipids were extracted and resolved on TLC plates and [^{32}P]-labeled phosphoinositides were measured by exposing the plates to phosphor screens. (1) acetylcholine; (2) acetylcholine + wortmannin (1 μM); (3) acetylcholine + wortmannin (100 nM); (4) acetylcholine + 10 nM; (5), acetylcholine + LY-294002 (10 μM); (6) acetylcholine + Y-27632 (1 μM); (7) acetylcholine + Y-27632 (10 μM); (8) untreated.

quantitated by densitometric measurement. We found that acetylcholine increased formation of phosphatidylinositide (3)P by 21–35.5 fold as compared to the untreated muscle strips. It appears that wortmannin used at the highest concentration can completely block D-3 phosphatidylinositide formation. In strips treated with lower concentrations of wortmannin partial inhibition of phosphatidylinositol 3-kinase activity was achieved (71,009 ± 8876, acetylcholine vs. 38,873 ± 3001, wortmannin 10 nM, $p = 0.00036$; 71,009 ± 8876 vs. 49,989 ± 9987, wortmannin 100 nM, $p = 0.00038$; densitometric counts) (lanes 3, 4). Specific phosphatidylinositol 3-kinase inhibitor, LY-294002, also completely blocked formation of phosphatidylinositide (3)P (lane 5). However, RhoA-kinase inhibitor, Y-27632 (1–10 μM) failed to have any significant effect on acetylcholine-induced phosphatidylinositide (3)P formation (71,009 ± 8875, acetylcholine vs. 59,099 ± 10,779, Y-27632, 1 μM; 71,009 ± 8875, acetylcholine vs. 68,993 ± 9989, Y-27632, Y-27632, 10 μM) (lanes 6, 7).

The ability of acetylcholine to induce phosphorylation of the p110 subunit of phosphatidylinositol 3-kinase was examined. Fig. 5 shows that acetylcholine induces phosphorylation of p110 as identified by immunoprecipitation/Western blot. The relative phosphorylations of the different strips were quantified by densitometric measurements. Acetylcholine treatment of muscle strips increased phosphorylation of p110 by 476 ± 11% as compared to control. Wortmannin (10 nM–1 μM) was added immediately prior to agonist treatment. Wortmannin at 10 and 100 nM decreased phosphorylation 38 ± 3% and 53 ± 5% compared to the acetylcholine-induced group. The highest concentration of wortmannin (1 μM) completely inhibited the phosphorylation process. We also checked the whether RhoA-kinase inhibitor, Y-27632, had any effect on acetylcholine-induced phosphorylation of phosphatidylinositol 3-kinase p110 subunit. Y-27632 had no effect (465,521 ± 33,456, acetylcholine vs. 486,255 ± 65,811, Y-27632, densitometric counts, not significant).

In other experiments, we examined whether Rho kinase, a downstream target of Rho, participates in acetylcholine- or PMA-induced phospholipase D activity. Rho-kinase has been shown to be a necessary downstream target for RhoA (Fu et al., 1998; Kuwahara et al., 1999; Sahai et al., 1999), and was inhibited by Y-27632 a relatively selective inhibitor of Rho-kinase (Kuwahara et al., 1999; Uehata et al., 1997). To compare relative contributions of different pathways leading to phospholipase D activation a series of experiments were performed on acetylcholine- and PMA-induced phospholipase D activity using various combinations of inhibitors. Data from these experiments are presented in Table 1. The inhibitors for RhoA-kinase (Y-27632, 1 μM) and protein kinase C (calphostin C, 1 μM) both independently inhibited acetylcholine-induced [^{3}H]phosphatidylethanol formation significantly when compared to acetylcholine alone. In general, combinations of inhibitors were not significantly more effective compared to any of the inhibitors used alone. However, blocking protein kinase C and RhoA-kinase pathways had the most significant effect (acetylcholine vs. Y-27632 + acetylcholine + calphostin C, $p = 0.00166$) on acetylcholine-induced [^{3}H]phosphatidylethanol formation. The finding that PMA-induced [^{3}H]phosphatidylethanol formation was not affected by Y-27632, wortmannin or

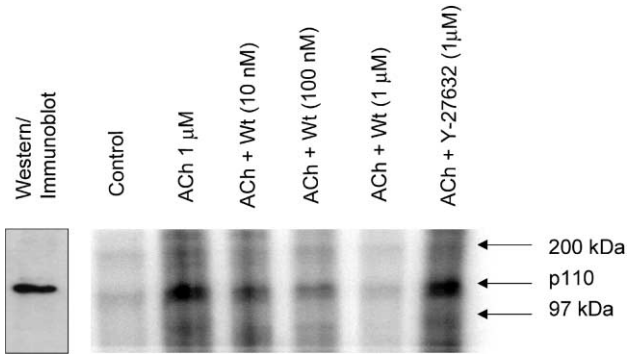

Fig. 5. Representative autoradiogram (one of three independent experiments) of effect of wortmannin and Y-27632 on the in vivo phosphorylation of phosphatidylinositol 3-kinase p110 subunit by acetylcholine. Muscle strips were labeled with [^{32}P]orthophosphate. Labeled strips were incubated with acetylcholine (1 μM) for 5 min. Where appropriate, wortmannin (10 nM–1 μM) or Y-27632 (1 μM) were added immediately prior to addition of acetylcholine. Phosphatidylinositol 3-kinase p110 was immunoprecipitated as described in Section 2. Immunoprecipitated proteins were then separated on a 10% SDS-polyacrylamide gel. Gels were dried, and phosphorylated proteins were detected by placing the gels on phosphor screen for 3 h and then scanning the exposed plate with a PhosphorImager.

Table 1
Effect of various combinations of inhibitors of Rho-kinase, phosphatidylinositol 3-kinase and protein kinase C on acetylcholine- and PMA-induced formation of [^{3}H]Phosphatidylethanol

Pretreatment	Treatment	[^{3}H]Phosphatidylethanol (cpm/Mg protein)
None	Control	3912 ± 209
None	Acetylcholine	14,755 ± 2156
Y-27634	Acetylcholine	8649 ± 332 *
Calphostin C	Acetylcholine	6627 ± 119 *
Y-27632 + Wortmannin	Acetylcholine	7985 ± 437 *
Y-27632 + Calphostin C	Acetylcholine	5500 ± 252 *
Wortmannin + Calphostin C	Acetylcholine	7274 ± 197 *
None	Y-27632	3529 ± 349
None	Control	3912 ± 362
None	PMA	10,329 ± 470
Y-27632	PMA	10,591 ± 716
Y-27632 + Wortmannin	PMA	10,780 ± 171
Y-27632 + Calphostin C	PMA	4214 ± 582 * *
Calphostin C	PMA	4145 ± 129 * *

[^{3}H]palmitic acid-labeled tracheal smooth muscle strips were preincubated in the presence of 100 mM ethanol for 10 min before exposing to acetylcholine (1 μM) or PMA (1 μM). Inhibitors (Y-27632, 1 μM; wortmannin, 1 μM; calphostin C, 1 μM) were added immediately prior to agonist treatment. All incubations were stopped after 5 min. Values are presented as means ± S.E.M. from three independent experiments.

* $p < 0.01$ compared with acetylcholine-treated group.

** $p < 0.01$ compared with PMA-treated group.

wortmannin and Y-27632 combined indicates that phosphatidylinositol 3-kinase is not involved in activation of phospholipase D via PMA-induced protein kinase C activation. Interestingly, calphostin C alone or in combination with Y-27632 both had similar and significant inhibitory effects on PMA-induced [^{3}H]phosphatidylethanol formation ($p = 0.00195$ and $p = 0.00122$, respectively).

4. Discussion

We have recently shown that acetylcholine transiently activates phospholipase D in porcine tracheal smooth muscle. Acetylcholine-induced phospholipase D activity appears to be partially dependent on protein kinase C activation since phospholipase D activation is only partially blocked by protein kinase C inhibitors in this tissue (Mamoon et al., 1999a). The finding suggested that pathway(s) other than protein kinase C might be involved in the activation of phospholipase D consequent to muscarinic receptor activation. In the present studies, we investigated the possible role of phosphatidylinositol 3-kinase as an additional pathway in muscarinic-induced phospholipase D activation. Phosphatidylinositol 3-kinase is considered to be a key signaling enzyme in a variety of receptor-stimulated signaling pathways (Katada et al., 1999). Stimulation of phosphatidylinositol 3-kinase generates several phosphorylated phosphoinositides PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3, all of which have been demonstrated to be second messengers (Stephens et al., 1993; Toker and Cantley, 1997), and PI(3,4)P_2 and PI(3,4,5)P_3 have been implicated in the activation of phospholipase D (Frohman et al., 1999). Tow selective inhibitors of phosphatidylinositol 3-kinase, wortmannin (Powis et al., 1994) and LY-294002 (Ibitayo et al., 1998), were used in these experiments. Both wortmannin and LY-294002 partially blocked acetylcholine-induced phospholipase D activation. Previously wortmannin has been claimed to be a phospholipase D inhibitor although no explanation regarding its mechanism has been provided (Carrasco-Marin et al., 1994). However, complete inhibition of phospholipase D activation could not be achieved with concentrations of wortmannin up to 1 μM. The fact that high concentrations of wortmannin did not completely inhibit phospholipase D activation suggests that wortmannin inhibits an activator of phospholipase D and does not inhibit phospholipase D directly.

In addition to our findings (Mamoon et al., 1999a) in porcine tracheal smooth muscle cells, it is well established that protein kinase C serves as a major regulator of phospholipase D activity in nearly all mammalian cells (Kiss, 1996). The tumor promoting phorbol ester, PMA, has been shown to selectively bind to and activate protein kinase C (Evans et al., 1991). Therefore, PMA has been routinely used to study the role of protein kinase C in various signal transduction pathways. PMA was used in the present studies to determine if protein kinase C-dependent phospholipase D activation in tracheal smooth muscle is mediated via activation of phosphatidylinositol 3-kinase. Wortmannin (10 nM–1 μM) or LY-294002 (10 μM) had no effect on PMA-induced phospholipase D activity in this tissue. The lack of effect of wortmannin or LY-294002 implies that phospholipase D activation via protein kinase C is not influenced by phosphatidylinositol 3-kinase activation in tracheal smooth muscle. Therefore, it appears that acetylcholine-induced phospholipase D activation in porcine tracheal smooth muscle occurs via both phosphatidylinositol 3-kinase-dependent and phosphatidylinositol 3-kinase-independent pathways.

We also demonstrated that acetylcholine activates phosphatidylinositol 3-kinase in tracheal smooth muscle and wortmannin or LY-294002 effectively block the activation of phosphatidylinositol 3-kinase. Wortmannin used at 1 μM and LY-294002 at 10 μM completely blocked phosphatidylinositol 3-kinase-induced incorporation of [^{32}P] into D-3 positions on phosphatidylinositols. It also appears that acetylcholine treatment causes the phosphorylation of a 110-kDa protein, which is immunoprecipitated with an antibody against the p110 catalytic subunit of phosphatidylinositol 3-kinase. Wortmannin also blocked the phosphorylation of p110 in a concentration dependent manner. The

findings suggest that acetylcholine-induced activation of phosphatidylinositol 3-kinase in tracheal smooth muscle is achieved by phosphorylation of the p110 catalytic subunit of phosphatidylinositol 3-kinase. It has been previously reported that muscarinic receptor agonists cause the translocation of the cytosolic small G protein, RhoA, from cytosol to plasma membranes, thus activating it (Baldassare et al., 1997; Keller et al., 1997). It has also been demonstrated that RhoA can activate phospholipase D (Abousalham et al., 1997; Karnam et al., 1997; Schmidt et al., 1999). Moreover, phosphatidylinositol 3-kinase has been shown to be involved in agonist-induced phospholipase D activation (Kozawa et al., 1997). In our studies, acetylcholine-stimulated translocation of RhoA to the membrane fractions was effectively blocked by wortmannin or LY-294002. This implies that activation of RhoA is also regulated by phosphatidylinositol 3-kinase. Since acetylcholine-induced phospholipase D activation is also blocked by wortmannin or LY-294002, it appears that phosphatidylinositol 3-kinase is an upstream regulator of both RhoA activation and phospholipase D activation in tracheal smooth muscle. We also demonstrated that acetylcholine-induced phospholipase D activity is dependent on Rho kinase since Rho kinase inhibitor, Y-27632, effectively blocks phospholipase D activation by acetylcholine. However, phosphatidylinositol 3-kinase activity as detected by phosphorylation of phosphatidylinositol was not blocked by Y-27632. This also supports the notion that phosphatidylinositol 3-kinase is an upstream regulator of RhoA activation.

While the mechanism of phospholipase D activation in smooth muscle is not yet confirmed, there is implication in nervous tissue (Morash et al., 2000) and human neutrophils (Lowe et al., 1996; Marcil et al., 1997) that activation of phospholipase D is a phosphorylation-dependent process. It is possible that a similar step of phosphorylation is mediated by Rho-kinase in activation of phospholipase D in tracheal smooth muscle since Rho-kinase inhibition partially blocks phospholipase D activation in this tissue. However, Y-27632 had no effect on PMA-induced phospholipase D activity, and also, inhibition of protein kinase C did not block translocation of RhoA to membrane in this tissue. The findings imply that at least the pathway acetylcholine → protein kinase C → phospholipase D (Mamoon et al., 1999a) is not regulated by phosphatidylinositol 3-kinase or RhoA activation in porcine tracheal smooth muscle. This is also supported by the findings that calphostin C alone blocks acetylcholine-induced [^{3}H]phosphatidylethanol as well as the combination of Y-27632 and wortmannin. Also PMA-induced [^{3}H]phosphatidylethanol is blocked by calphostin C alone to a similar extant as Y-27632 and calphostin C combined (Table 1). However, it appears that these pathways do not work totally parallel since the combined effects of the inhibitors are not significantly different when compared to effects of the inhibitors alone. Thus they are not additive in their effects. Rather, several separate pathways possibly converge at some point in the phosphatidylinositol 3-kinase and protein kinase C pathways. It is possible that among the multiple known pathways involved in phospholipase D activation consequent to muscarinic agonist stimulation, the pathway involving protein kinase C plays the principal role.

Recently, the RhoA/Rho kinase pathway has been implicated in the process of Ca^{2+} sensitization in agonist-induced contraction in smooth muscle (Somlyo and Somlyo, 2000). However, the mechanism for activating RhoA have not been well described. In these experiments, we demonstrate a link between muscarinic receptor-mediated phosphatidylinositol 3-kinase activation, translocation of RhoA to the membrane and RhoA kinase activation implying a possible pathway for muscarinic-induced Ca^{2+} sensitization. RhoA has also been shown to be involved in several important cellular functions including cell adhesion, stress fiber formation, Ca^{2+} release and cell motility (Kosako et al., 2000). Since phosphatidic acid has been suggested to be involved in activation of RhoA (Lee et al., 1998), and activation of phospholipase D in tracheal smooth muscle generates phosphatidic acid (Mamoon et al., 1999a,b) we speculate that phosphatidic acid may be essential for maintaining the sustained activity of RhoA in the cell. In favor of this view, it has been shown that phosphatidic acid, along with other phospholipids, dissociate Rho-GDI complex in in vitro (Chuang et al., 1993). RhoA exists as a biologically inactive GDP-bound form in the cytosol complexing with Rho-GDI (guanine nucleotide exchange inhibitor) and needs to be dissociated from the complex before it can be activated (Longenecker et al., 1999). We have demonstrated that stimulation of tracheal smooth muscle by cholinergic agents can increase phosphatidic acid formation via both phosphatidylinositol 3-kinase-dependent and phosphatidylinositol 3-kinase-independent pathways and it is possible that multiple pathways leading to phosphatidic acid formation helps to maintain an optimal level of phosphatidic acid following muscarinic stimulation of tracheal smooth muscle even though the direct activation of phospholipase D appears to be transient (Mamoon et al., 1999a). This view is very speculative since phosphatidic acid-induced dissociation of Rho-GDI in intact muscle strips has not yet been demonstrated.

In summary, we have demonstrated that muscarinic receptor agonist-induced activation of phospholipase D in tracheal smooth muscle is at least partly dependent on phosphatidylinositol 3-kinase activation, which is dependent on wortmannin- or LY-294002-sensitive phosphorylation of the p110 catalytic subunit of phosphatidylinositol 3-kinase. Phosphatidylinositol 3-kinase-dependent stimulation of phospholipase D in this tissue is also regulated by activation of RhoA; but, phosphatidylinositol 3-kinase appears to be an upstream regulator of RhoA. As previously shown, a separate pathway also exists for acetylcholine-induced phospholipase D activation requiring protein kinase C activation, however, this pathway is not influenced by either phospha-

tidylinositol 3-kinase or RhoA activation. To better define the molecular events in this pathway, the isoforms of both phosphatidylinositol 3-kinase and phospholipase D, translocation/activation or phosphorylation status of the isoform(s) on muscarinic stimulation of intact muscle strips need to be studied.

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