Acute Experimental Esophagitis Activates a Second Signal Transduction Pathway in Cat Smooth Muscle from the Lower Esophageal Sphincter


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ABSTRACT

In single cells, isolated by enzymatic digestion from the circular muscle layer of the lower esophageal sphincter (LES), acute experimental esophagitis (AE) alters signal transduction in response to a maximally effective dose of acetylcholine. In normal LES contraction was inhibited by M3 >> M1 or M2 antagonists. In AE inhibition by M2 antagonists increased significantly so that contraction was inhibited by M3 > M2 > M1 antagonists. In normal cells permeabilized by saponin, contraction was antagonized by antibodies against Gα11, by the phosphatidylinositol-specific phospholipase C (PI-PLC) antagonist U73122, but not by the phosphatidylinositol-specific phospholipase C (PC-PLC) inhibitor D609, or by the phospholipase D pathway inhibitor propranolol. In AE contraction was reduced by Gα11 and Gα3 antibodies and by U73122, propranolol and D609. After thapsigargin treatment of normal cells to reduce intracellular Ca2+ stores, contraction was inhibited by M1 and M3 antagonists, by antibodies against Gα11 and Gα3, by U73122, D609 and propranolol, suggesting that depletion of Ca2+ stores reproduces the changes induced by AE. We conclude that in normal LES smooth muscle cells acetylcholine-induced contraction is mediated by M1 receptors linked to Gα11 and PI-PLC, whereas in AE, contraction through this pathway is reduced, perhaps because of reduction in Ca2+ stores, and a second pathway is activated by M3 receptors linked to Gα3, PC-PLC and phospholipase D.

Two distinct contractile signal transduction pathways are present in LES muscle cells. A PI-PLC, 1,4,5-IP3, calmodulin-dependent pathway is activated by stimulation with a maximally effective dose of ACh. In this pathway M3 muscarinic receptors, linked to Gα11-type G-proteins, stimulate PLC, which results in the formation of 1,4,5-IP3 and DAG. 1,4,5-IP3 causes the release of Ca2+ from intracellular stores producing a calcium-calmodulin complex, myosin light chain phosphorylation and contraction (Biancani et al., 1994). This pathway is PKC-independent, because activated calmodulin may inhibit PKC activity (Biancani et al., 1994; Chakravarthy et al., 1995a, b; Kraft and Anderson, 1983; Yu et al., 1993; Zhao et al., 1991).

A second PKC-dependent pathway is activated by submaximal doses of ACh or during maintenance of LES tone. In this pathway, submaximal doses of ACh or spontaneous tone are linked to low levels of PLC activity, resulting in low levels of 1,4,5-IP3 which causes the release of low levels of Ca2+ from intracellular stores. These low Ca2+ levels are insufficient to activate calmodulin but can act synergistically with DAG to activate PKC (Biancani et al., 1994). Thus the amount of Ca2+ available for contraction determines which pathway will be followed, with low Ca2+ levels activating a PKC-dependent pathway, and high levels activating a calmodulin-dependent pathway.

In a model of AE, repeated perfusion of the esophagus with 0.1 N hydrochloric acid causes a reduction in resting in vivo LES pressure, in vitro spontaneous tone and levels of 1,4,5-IP3 (Biancani et al., 1984). These data suggest that AE affects...
zymes localized to the membrane of isolated smooth muscle depends on different receptors, G-proteins and effector environment. Thapsigargin, a PKC-dependent pathway is activated, which depends on different receptors, G-proteins and effector enzymes localized to the membrane of isolated smooth muscle cells of the LES.

Materials and Methods

Animals. Experimental procedures were approved by the animal welfare committee of Rhode Island Hospital. Adult cats of either sex, weighing 3 to 5 kg, were used in this study. After an overnight fast, they were anesthetized with ketamine (10 mg/kg), and maintenance doses of ketamine (2.5–5 mg/kg) were administered as needed. To determine LES position, esophageal pressure was measured by a repeated station pull-through technique, 1 to 2 mm at the time, with a multilumen catheter having three proximal openings 3 cm apart and a distal perfused sleeve. The sleeve device was used to measure LES pressure, whereas the three proximal openings measured amplitude of contraction in the body of the esophagus. With the sleeve placed across the LES, the most proximal opening, which was 9 cm proximal to the LES, was used to perfuse acid in the esophagus when needed.

In each experiment two different groups of animals were examined: the first group consisted of normal animals, whereas animals from the second group (esophagitis animals), after initial measurement of LES pressure, had their esophagus perfused with 0.1 N HCl at a rate of 1 ml/min for 45 min during 3 consecutive days. These animals were placed on a slant board at a 30° angle during the perfusion to avoid aspiration. This protocol has been shown to produce inflammatory changes in the esophageal mucosa and submucosa. Our previous histologic studies with this model of experimental esophagitis show no evidence of inflammatory infiltrate in the circular muscle layer, which appears normal under light microscopy, and under electron microscopy exhibits changes (enlargement of mitochondria and slight disruption of endoplasmic reticulum) characteristic of an early inflammatory phase (Biancani et al., 1994). There is a significant decrease of spontaneous G-protein remnants in the LES in vitro resting pressure and in vitro spontaneous tone, whereas esophageal perfusion with distilled water had no effect on mucosal appearance or LES resting pressure (Biancani et al., 1984; Eastwood et al., 1976).

Tissue preparation. The animals were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, N. Chicago, IL) (50 mg/kg) and the chest and abdomen were opened with a midline incision, exposing the stomach, esophagus and esophagogastric junction. A suture was placed on the esophagogastric junction, and a second suture was placed on the esophageal body, 5 cm proximal to it. Stomach, esophagogastric junction and esophagus were removed together and pinned on a wax block, with the distance between the sutures held at 5 cm. This insured that the specimen was stretched to its in vivo length. The esophagus and stomach were opened along the lesser curvature. The location of the squamocolumnar junction was identified, and the mucosa and submucosa were removed by sharp dissection under microscope. The high-pressure zone of the LES is characterized by a visible thickening of the circular muscle layer at the squamocolumnar junction, and immediately proximal to the sling fibers of the stomach. We previously showed that a 5- to 8-mm band of tissue coinciding with the thickened area constitutes the LES and has distinct characteristics when examined in vitro in the organ bath, or as single cells after enzymatic digestion (Biancani et al., 1982,1987). After surgically isolating the LES, it was placed, serosal side down, in a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA) and cut into 0.5-mm-thick slices. The last slices containing the myenteric plexus, longitudinal muscle and serosa were discarded. This method provided approximately 600 to 800 mg of relatively pure circular smooth muscle of LES. The slices of smooth muscle were placed flat on a wax surface and tissue squares were made by cutting twice with a 2-mm blade block, the second cut at right angles to the first. LES circular smooth muscle squares were used for measurement of PKC activity and 1,4,5-IP3 formation, or further digested to isolated single cells for contractility studies.

Cell dispersion. Isolated smooth muscle cells were obtained by enzymatic digestion, as described previously (Biancani et al., 1987). LES smooth muscle squares were digested in HEPES-buffered physiologic solution, containing collagenase 150 U/ml (CLS type II, Worthington Biochemicals, Freehold, NJ) for 2 h. The HEPES solution contained: NaCl, 114.7 mM; KCl, 5.7 mM; KH2PO4, 2.1 mM; glucose, 11 mM; HEPES, 24.5 mM; CaCl2, 1.9 mM; MgCl2, 0.57 mM, BME amino acid supplement (M.A. Bioproducts, Walkersville MD) 0.3 mg/ml, and soybean trypsin inhibitor (Worthington Biochemicals, Freehold, NJ) 0.08 mg/ml. The HEPES solution was oxygenated (100% O2) at 31°C, and the pH was adjusted to 7.4. During the digestion period the gas flow rate was kept low to avoid agitating the tissue. At the end of the digestion period, the tissue and digestion medium were poured out over 500-μm Nitex mesh (Tetko, Inc., Elmsford, NY). The tissue on the mesh was rinsed with 150 ml of collagenase-free HEPES solution to remove any trace of collagenase and then incubated in collagenase-free HEPES solution at 31°C. The cells were allowed to dissociate freely in the collagenase-free solution for 10 to 20 min. Care was taken not to agitate the fluid to avoid cell contraction in response to mechanical stress.

All the glassware used in this procedure was prerinsed in a 0.05% silicon solution to prevent the cells from adhering to the glass.

Permeabilization of single cells. Isolated LES smooth muscle cells were permeabilized, when required, to allow the use of G-protein antibodies, which usually do not diffuse across the intact plasma membrane, or to control the intracellular calcium concentration. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with a “cytotoxic” enzyme-free physiologic salt solution (cytotoxic buffer) of the following composition (mM): NaCl, 20; KCl, 100; MgSO4, 5.0; NaH2PO4, 0.96; EGTA, 1.0; and CaCl2, 0.48. The cytotoxic buffer contained 2% bovine serum albumin and was equilibrated with 95% O2/5% CO2 to maintain a pH of 7.2 at 31°C. Muscle cells dispersed spontaneously in this medium. Permeabilization was accomplished by incubation of the freely dispersed cells for 3 min in cytotoxic buffer containing saponin (75 μg/ml). When this incubation was complete, the cell suspension was spun at 500 × g and the pellet resuspended in saponin-free modified cytotoxic buffer containing antimycin A (10 μM), ATP (1.5 mM) and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase (10 U/ml) (Bitar et al., 1986). The procedure was repeated twice to ensure complete removal of saponin. After the cells were washed free of saponin, they were resuspended in the modified cytotoxic buffer.

Experimental procedure. Once the cells had dissociated, 0.5-m1 aliquots of the cell-containing fluid were added to tubes for exposure to agonists and measurement of contraction. Cells were exposed to a maximally effective dose of ACh (10−10 to 10−6 M) for 30 s (Hillemeier et al., 1991). When antagonists were used, cells were incubated in appropriate concentration of the antagonist for 1 min before the addition of ACh. When G-protein antibodies were used, the permeable cells were incubated in the antiserum at a final concentration of 1 to 2 μg/ml for 1 h before the addition of ACh. When thapsigargin-induced reduction in intracellular Ca2+ stores, cells were preincubated in 10−6 M thapsigargin for 30 min before the addition of ACh and/or agonists.

After exposure to ACh, the cells were fixed in acrolein at a final 1% concentration. A drop of the cell-containing medium was placed...
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on a glass slide and covered by a cover slip. The edges of the cover slip were sealed with clear nail enamel to prevent evaporation. Slides so prepared, if refrigerated, could be kept for several days.

Cell measurements. Thirty consecutive cells from each slide were observed through a phase-contrast microscope (Carl Zeiss, Oberkorn, Germany), and a CCTV camera (model WV-CD51, Panasonic, Secaucus, NJ) connected to a Macintosh IIci Computer (Apple, Inc., Cupertino, CA). The Image 1.33 software program (NIH, Bethesda, MD) was used to measure cell length and for data accumulation. The average length of 30 cells, measured in the absence of agonists, was taken as “control” length. In addition, average cell length was measured after addition of test agents. Control cells underwent the same manipulations as stimulated cells. Shortening was defined as the percent decrease in average length after agonists, when compared with control length.

Measurement of PKC activity. Measurement of PKC activity in the cytosolic and membrane fraction, was performed by colorimetric assay. The cytosolic and membrane fraction of LES circular smooth muscle were prepared from normal cats and from cats after the induction of AE as follows. LES smooth muscle squares (600–800 mg) obtained from one cat were equilibrated in 400 µl Krebs’ solution and gassed with 95% O2-5% CO2 at 37°C for 20 min. For measurement of agonist-stimulated PKC activity LES smooth muscle squares were divided into 150-mg aliquots. One was used for PKC measurement of a control (untreated) sample, a second was exposed to ACh (10^{-7} M) and a third was exposed to ACh (10^{-5} M); then PKC activity was measured. ACh (10^{-5} M) was previously determined to produce maximal contraction in LES smooth muscle strips. After 30 s the reaction was stopped with 10 volumes of ice-cold Krebs’ solution. Muscle squares were collected and homogenized in 20 mM Tris buffer, pH 7.4, containing: EDTA, 0.5 mM; EGTA, 0.5 mM; leupeptin, 10 mg/ml; aprotinin, 10 mg/ml, and β-mercaptoethanol, 10 mM. Homogenization consisted of 2- to 10-s bursts with a Tissue Tearer (Biospec, CA). The supernatant was collected, and after the addition of 30 µl of homogenizing buffer containing 0.1% Triton X-100 and rehomogenization consisted of 2- to 10-s bursts with a Tissue Tearer (Biospec, Racine, WI) followed by 40 to 60 strokes with a Dounce tissue grinder (Wheaton, Melville, NJ). Samples were centrifuged at 100,000 g for 40 min at 4°C, and centrifuged at 100,000 g for 50 min at 4°C. The resultant supernatant was collected as the membrane fraction. The cytosolic and membrane fractions were immunoprecipitated by isozyome-specific PKC antibodies. We previously reported that, in the LES circular smooth muscle, contraction is mediated by the PKCβII isoform (Sohn et al., 1997). Five micrograms of antibodies raised against PKCβII were added to 400 µl of cytosolic or membrane fraction, and incubated for 1 h at 4°C. Forty microliters of protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and samples were incubated at 4°C with rocking. After 1 h samples were microcentrifuged (Microcentrifuge, Fisher Scientific, Pittsburgh, PA) for 15 to 20 s at 4°C. The pellet was washed in 20 µl RIPA buffer and solubilized. The supernatant was removed, and the pellet was suspended in 20 µl RIPA buffer containing: KH2PO4, 1 mM; Na2HPO4, 10 mM; NaCl, 137 mM; KCl, 2.7 mM; 1% tritertol, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4 and solubilized. Ten microliters of solubilized sample was used in the PKC colorimetric assay.

PKC activity of immunoprecipitated smooth muscle of the LES was measured by the Pierce Colorimetric PKC Assay No. 29510 (Pierce, Rockford, IL). A peptide substrate, labeled with a brightly colored fluorescent dye, was incubated with the kinase-containing sample. The reaction mixture was applied to an affinity column that binds phosphorylated peptides. The phosphorylated product was eluted from the column and quantitated by measurement of its absorbance at 570 nm.

Measurements of inositol phosphates. The method for the measurement of inositol phosphates, by HPLC has been described previously (Biancani et al., 1992; Szewczak et al., 1990). LES circular smooth muscle squares were incubated for 4.5 h (37°C) in 1.5 ml Krebs’ solution containing 60 µCi/µmol ymo-[2-3H]inositol and gassed with 95% O2-5% CO2. After incubation, the smooth muscle squares were poured out over 360-µl Nitex mesh (Tetko Inc, Elmsford NY) and rinsed with 50 ml of Krebs’ solution to remove excess [2-3H]inositol. The muscle squares were evenly divided into the experimental tubes containing 0.5 ml of gassed Krebs’ solution at 37°C and allowed to equilibrate for 30 min at 37°C. Krebs’ solution (control; 0.5 ml) or Krebs’ solution containing 10^{-5} M ACh was then added and the experiment stopped at the appropriate time points by the addition of 0.6 ml of chloroform/methanol/HCl (100:50:1) and 25 µl phytate (100 mg/ml) (Hughes et al., 1988). Samples were collected at zero time and at 1 and 5 s. Zero time levels were obtained by adding the vehicle only (0.5 ml of Krebs’ solution) and immediately quenching the tissue. The aqueous, cytosolic phase from each sample was separated by centrifugation and 0.5 ml was removed. The remaining aqueous phase was washed twice with ice-cold water and a total 1.5 ml of aqueous extract was collected and neutralized to pH 6.5 to 7.5 with 0.5 N NaOH. The extract was stored at −70°C for later analysis. The remaining tissue-containing aqueous and organic phases were stored at −70°C for protein determination.

The aqueous sample was applied to a Partisil 10 SAX HPLC column (Whatman, Clifton, NJ). Inositol phosphate metabolites were separated with an ammonium formate gradient (pH 3.7; flow rate, 1.2 ml/min) as described previously (Biancani et al., 1992; Szewczak et al., 1990). After 2.5 min of water, the concentration of ammonium formate was increased to 3% for 21 min, 23% for 9 min, 50% for 9 min followed by the final gradient increase to 100% ammonium formate for 15 min.

Radioactivity was determined by continuous flow liquid scintillation counting with a FLO-ONE\Beta Radioschromatography Detec- tor System (Radiomatic Instruments and Chemicals Co., Inc, Tampa, FL) with FLO-SCINT IV scintillator (Packard Instrument Company, Inc., Downer's Grove, IL). The data were converted to dpm with use of the counting efficiency of an [2-3H]1,4,5-IP3 standard (Amersham, Arlington Heights, IL). This efficiency averaged 37.4% and was constant throughout the elution gradient. 1-IP3, 1, 4-IP3 and 1,4,5-IP3 were identified by use of standard [2-3H]inositol phosphate metabolites. In all cases, the inositol phosphate metabolites eluted as sharp peaks. The data were expressed as dpm/mg protein.

Protein determination. Protein content was obtained after hydrolysis by 0.1 N NaOH at 80°C to solubilize the protein, followed by neutralization with HCl. The amount of protein present was determined by colorimetric analysis (BioRad Protein Assay; Bio Rad Labo- ratories, Richmond CA) according to the method of Bradford (1976).

Drugs and chemicals. G-protein antibodies (Gαi1, Gαq, Gαs, Gα12, G12-13) raised against synthetic peptides corresponding to the amino acid sequence of the COOH-terminal of the G-protein α subunits were purchased from New England Nuclear (Boston, MA); methoc- tramine HCl, pirenepine 2 HCl and p-F-HSD from Research Biochemical Inc (Natick, MA); collagenase type II and soybean trypsin inhibitor from Worthington Biochemicals (Freehold, NJ); D609 from Kamiya Biochemical Co (Thousand Oaks, CA); H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperezine dihydrochloride) from Seikagaku America Inc. (St. Petersburg FL) and U73122 from Biomol (Plymouth Meeting, PA). Myo-[2-3H]inositol and [2-3H]inositol phosphate metabolites were purchased from Amersham Corp.(Arlington Heights, IL). CGS9343B was a gracious gift of Dr. M. Crettaz from Zyma SA, NYON, Switzerland.

Where not otherwise mentioned, all other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).
Statistical analysis. Data are expressed as the mean ± S.E. Statistical differences between multiple groups were tested by analysis of the variance (ANOVA) for repeated measures and checked for significance with use of the Scheffe’s F-test.

Results

AE causes a switch in the intracellular contractile pathway mediating ACh-induced contraction. We examined PKC translocation from the cytosol to the membrane in response to stimulation of LES circular smooth muscle squares by ACh. Translocation of the enzyme from the cytosol to the membrane fraction is thought to be a measure of PKC activation (Khalil and Morgan, 1991; Kraft and Anderson, 1983; Nishizuka, 1986, 1989). Figure 1A shows that in normal LES tissue PKC activity (measured by membrane/cytosolic PKC activity ratio) increases significantly at first (ANOVA, P < .05), then decreases when a maximally effective concentration (10⁻⁶ M) of ACh is used. In contrast, after induction of AE, PKC activity increases dose-dependently with ACh to a maximum 3-fold increase at 10⁻⁶ M ACh (ANOVA, P < .001). Thus it is likely that, at least at maximally effective ACh concentration (10⁻⁶ M), PKC may mediate contraction in AE but not in normal LES.

We previously showed that LES resting 1,4,5-IP₃ levels are reduced after induction of AE (Biancani et al., 1992). To test whether stimulated 1,4,5-IP₃ levels are similarly reduced, we measured inositol phosphate production in LES circular smooth muscle squares in response to ACh with HPLC. One and five seconds after the administration of ACh, 1-IP₁, 1,4-IP₂, and 1,4,5-IP₃ increased significantly in the normal LES (ANOVA, P < .05); but not in AE muscle (fig. 1B). After 1 and 5 s exposure to ACh, 1,4,5-IP₃ increased 59.4% and 82.8%, respectively, above the unstimulated value in normal LES. However, 1-IP₁, 1,4-IP₂, and 1,4,5-IP₃ levels of AE LES did not increase with maximal ACh stimulation. This finding supports the view that ACh-induced stimulation is associated with a significant increase in inositol phosphate production in normal LES, but not in AE.

The data presented in figure 1 suggest that, in the normal and AE LES muscle, different intracellular pathways may be activated in response to ACh. In the normal LES, ACh causes production of 1,4,5-IP₃; in contrast in AE, ACh causes PKC activation. The data are consistent with previous findings (Rich et al., 1997) that contraction induced by a maximally effective dose of ACh is mediated by a calmodulin-dependent pathway in normal LES and a PKC-dependent pathway after induction of AE. We therefore investigated whether the transduction mechanisms localized to the membrane, such as the receptors, G-proteins and effector enzymes, are affected by the induction of AE.

Different muscarinic receptors mediate ACh-induced contraction in normal LES cells and AE cells. Figure 2 shows the effect of the M₃ receptor antagonists pirenzepine, methoctramine and pF-HSD, respectively, on the contraction induced by a maximally effective dose of ACh (10⁻⁶ M) in normal LES smooth muscle cells and in AE cells. It should be noted that for single cell experiments the maximally effective ACh concentration is lower than when intact tissue is used. Cells were exposed for 1 min to 10⁻⁶ M antagonist before contraction with ACh. Figure 2 shows in normal smooth muscle cells ACh-induced contraction was reduced by all antagonists, but the M₃ antagonist pF-HSD caused the largest inhibition, reducing contraction by two thirds (ANOVA, ***P < .001). Table 1 shows that % inhibition of contraction after methoctramine pretreatment was significantly greater in AE than in normal LES (unpaired t test, P < .05). After induction of AE the M₃ antagonist still caused a two-thirds reduction in contraction (fig. 2, ANOVA, ***P < .001).

These data suggest that after esophagitis, ACh-induced contraction is no longer mediated predominantly by M₃ receptors but depends on activation of both M₂ and M₃ receptors.

Different G-proteins mediate ACh-induced contraction in normal LES cells and AE cells. Because AE affects the receptors responsible for ACh-induced contraction, it is reasonable to expect that the G-proteins linked to these receptors may be similarly affected. To identify the specific G-proteins, we used G-protein antibodies raised against synthetic peptides corresponding to the amino acid sequence of the COOH-terminal of the G-protein α subunit. The cells were permeabilized by brief exposure to saponin to allow diffusion of the antibodies into the cytoplasm, as described previously (Sohn et al., 1993, 1995).

Different phospholipases mediate ACh-induced contraction in normal and AE LES cells. The difference in G-proteins activated in normal and AE LES cells may cause differences in the phospholipases activated in response to maximally effective ACh. To test this hypothesis, cells from normal cats and from cats with esophagitis were contracted with ACh (10⁻⁹ M) in the presence of the phospholipase inhibitors, U73122 (10⁻⁶ M), D609 (10⁻⁴ M) and propranolol (10⁻⁴ M). U73122 inhibits PI-PLC (Bleasdale et al., 1989), D609 inhibits PC-PLC (Schutze et al., 1992) and propranolol in high concentrations inhibits phosphatidic acid phosphohydrolase, preventing formation of DAG through a PLD-mediated pathway (Billah et al., 1989; Qian and Drewes, 1990).

Figure 3 shows that contraction of normal cells was significantly inhibited by the G_{q/11} antibody (ANOVA, P < .001), and slightly inhibited by G_α₁₂, G_α_{i3} or G_α_{i3} antibodies. Contraction of esophagitis cells was significantly inhibited by antibodies raised against G_{q/11} and G_{α_{i3}} (ANOVA, P < .001) but not by G_α, G_{i1−2} or G_{i1−3} antibodies. These data suggest that ACh-induced contraction of normal LES depends on G_{q/11} activation, and after induction of AE contraction depends on both G_{q/11} and G_{i3}.

Different phospholipases mediate ACh-induced contraction in normal and AE LES cells. The difference in G-proteins activated in normal and AE LES cells may cause differences in the phospholipases activated in response to maximally effective ACh. To test this hypothesis, cells from normal cats and from cats with esophagitis were contracted with ACh (10⁻⁹ M) in the presence of the phospholipase inhibitors, U73122 (10⁻⁶ M), D609 (10⁻⁴ M) and propranolol (10⁻⁴ M). U73122 inhibits PI-PLC (Bleasdale et al., 1989), D609 inhibits PC-PLC (Schutze et al., 1992) and propranolol in high concentrations inhibits phosphatidic acid phosphohydrolase, preventing formation of DAG through a PLD-mediated pathway (Billah et al., 1989; Qian and Drewes, 1990).

Figure 4 shows that ACh-induced contraction of normal cells was significantly reduced by U73122 only (ANOVA, P < .001), and not by D609 or propranolol. In contrast, after induction of AE, ACh-induced contraction was significantly reduced by all three phospholipase inhibitors (ANOVA, P < .001), which suggests that all three enzymes, PI-PLC, PC-PLC and PLD, may mediate ACh-induced contraction.

M₃ receptors are linked to G_{q/11} and PI-PLC; M₂ receptors are linked to G_{α₃} and PC-PLC and PLD. After induction of AE, contraction induced by a maximally effective dose of ACh is mediated by M₂ and M₃ receptors, activating G_{q/11}, G_{α₃} and three phospholipases, PI-PLC, PC-PLC and PLD. To test whether two separate pathways exist, permeabilized smooth cells from esophagitis cats were incubated in a maximally effective concentration of antibodies raised against either G_{q/11} or G_{α_{i3}} for 1 h before contraction with ACh. In addition to the antibodies, cells were exposed to...
muscarinic or phospholipase antagonists 1 min before ACh administration.

Figure 5A shows that in the presence of a maximally effective concentration of Gq/11 antibodies (Sohn et al., 1993), ACh-induced contraction was not further reduced by the M3 antagonist pF-HSD or by the PI-PLC inhibitor U73122, which suggests that these antagonists inhibit the same signal transduction pathway that is linked to Gq/11. In contrast, the M2 antagonist methoctramine, the PC-PLC inhibitor

Fig. 1. (A) Maximal ACh causes PKC activation in AE LES but not in normal LES circular smooth muscle. In normal LES circular smooth muscle, PKC activity (measured by membrane-to-cytosolic PKC activity ratio) significantly increases at first (ANOVA, *P < .05), then decreases when a maximally effective concentration (10^-5 M) of ACh is used. In contrast, after induction of AE, PKC activity increases dose-dependently with ACh to a maximum three-fold increase at 10^-2 M ACh (ANOVA, ***P < .001). PKC activity was measured by colorimetric assay using cytosolic and membrane fractions of LES circular smooth muscle immunoprecipitated with PKCβII isozyme-specific antibodies (Sohn et al., 1997). Values shown are the means ± S.E. of three animals. (B) ACh causes production of inositol phosphates 1-IP1, 1,4-IP2 and 1,4,5-IP3 in normal LES circular smooth muscle (ANOVA, P < .05) but not in AE LES. After incubation in 60 μCi/ml myo-[2-3H]inositol for 4.5 h, LES smooth muscle was exposed to Krebs’ buffer (control) or ACh (10^-5 M) for 1 and 5 s. Aqueous fractions containing inositol phosphates were separated by HPLC and the integrated peaks of radioactivity were measured on-line. Values are the means ± S.E. of four to six experiments.

Fig. 2. ACh-induced contraction is mediated by M3 muscarinic receptors in normal LES cells, and by both M2 and M3 receptors in AE LES cells. Isolated smooth muscle cells of the circular layer of the LES were contracted by a maximally effective dose of ACh (10^-9 M) alone (control) or after 1 min pretreatment with 10^-6 M M2, or M3 muscarinic antagonists pirenzepine, methoctramine or pF-HSD, respectively. In normal smooth muscle cells ACh-induced contraction was reduced by all antagonists (table 1), but the M3 antagonist pF-HSD caused the largest inhibition, reducing contraction by two thirds (ANOVA, ***P < .001). After induction of AE, inhibition by the M2 antagonist increased. The M3 antagonist still caused a two-thirds reduction in contraction (ANOVA, ***P < .001). Values are means ± S.E. of three to four animals, with 30 cells counted for each animal.

TABLE 1

Percent Inhibition of contraction in response to a maximally effective dose of ACh (10^-9 M) by muscarinic antagonists pirenzepine (M1), methoctramine (M2) and pF-HSD (M3) in normal and acute experimental esophagitis LES smooth muscle cells

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<th>Muscarinic Antagonists (10^-6 M)</th>
<th>Normal LES</th>
<th>Esophagitis LES</th>
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<tr>
<td>Pirenzepine</td>
<td>29.00 ± 3.12</td>
<td>20.00 ± 3.43</td>
</tr>
<tr>
<td>Methoctramine^b</td>
<td>20.10 ± 4.57</td>
<td>42.07 ± 6.34</td>
</tr>
<tr>
<td>pF-HSD^c</td>
<td>70.00 ± 4.27</td>
<td>65.55 ± 1.59</td>
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^a Values represent mean ± S.E. of four cats. % inhibition of contraction by pirenzepine in normal and esophagitis cats, and by methoctramine in normal animals were relatively low and did not differ from each other. This inhibition may be related to nonselective binding of antagonists to muscarinic receptors.

^b Methoctramine-induced inhibition was significantly greater in AE than in normal LES (unpaired t-test, *P < .05).

^c pF-HSD caused the greatest inhibition in normal and esophagitis animals.

muscarnic or phospholipase antagonists 1 min before ACh administration.

Figure 5A shows that in the presence of a maximally effective concentration of Gq/11 antibodies (Sohn et al., 1993), ACh-induced contraction was not further reduced by the M3 antagonist pF-HSD or by the PI-PLC inhibitor U73122, which suggests that these antagonists inhibit the same signal transduction pathway that is linked to Gq/11. In contrast, the M3 antagonist methoctramine, the PC-PLC inhibitor phosphates 1-IP1, 1,4-IP2 and 1,4,5-IP3 in normal LES circular smooth muscle (ANOVA, P < .05) but not in AE LES. After incubation in 60 μCi/ml myo-[2-3H]inositol for 4.5 h, LES smooth muscle was exposed to Krebs’ buffer (control) or ACh (10^-5 M) for 1 and 5 s. Aqueous fractions containing inositol phosphates were separated by HPLC and the integrated peaks of radioactivity were measured on-line. Values are the means ± S.E. of four to six experiments.
ACh-induced contraction is mediated by G_{q/11} G proteins in normal LES cells, and by both G_{q/11} and G_{i3} in AE LES cells. Different G-proteins mediate ACh-induced contraction of permeabilized circular smooth muscle cells of normal LES and after induction of AE. Muscle cells were permeabilized by brief exposure to saponin to allow diffusion of antibodies into the cytosolic side of the cell membrane. Cells were contracted with ACh (10^{-9} M) alone (control) or after 60 min preincubation in cytosolic medium containing G-protein antibodies (1:200 dilution). ACh-induced contraction of normal LES cells was significantly inhibited by G_{q/11} (ANOVA, ***P < .001) and by G_{i1–2}, G_{i3}, or G_{i3} antibodies, whereas contraction of AE cells was inhibited by antibodies raised against both G_{i3} and G_{q/11} (ANOVA, ***P < .001) and not by G_{i1–2} or G_{i3}. These data suggest that G_{q/11} may mediate contraction of normal LES whereas both G_{q/11} and G_{i3} may mediate contraction of LES after the induction of AE. Values are the means ± S.E. of three to four animals with 30 cells counted at random for each data point.

Fig. 3. ACh-induced contraction is mediated by G_{q/11} and G_{i3} G-proteins in normal LES cells and, by both G_{q/11} and G_{i3} in AE LES cells. Different G-proteins mediate ACh-induced contraction of permeabilized circular smooth muscle cells of normal LES and after induction of AE. Muscle cells were permeabilized by brief exposure to saponin to allow diffusion of antibodies into the cytosolic side of the cell membrane. Cells were contracted with ACh (10^{-9} M) alone (control) or after 60 min preincubation in cytosolic medium containing G-protein antibodies (1:200 dilution). ACh-induced contraction of normal LES cells was significantly inhibited by G_{q/11} (ANOVA, ***P < .001) and not by G_{i1–2}, G_{i3} or G_{i3} antibodies, whereas contraction of AE cells was inhibited by antibodies raised against both G_{i3} and G_{q/11} (ANOVA, ***P < .001) and not by G_{i1–2} or G_{i3}. These data suggest that G_{q/11} may mediate contraction of normal LES whereas both G_{q/11} and G_{i3} may mediate contraction of LES after the induction of AE. Values are the means ± S.E. of three to four animals with 30 cells counted at random for each data point.

ACh-induced contraction of thapsigargin-treated cells was significantly reduced only by U-73122 in normal LES (ANOVA, ***P < .001). After induction of AE, ACh-induced contraction was significantly reduced by all three phospholipase inhibitors (ANOVA, ***P < .001).

Discussion

AE causes a switch in the intracellular contractile pathway mediating ACh-induced contraction. We have
calcium are low, as during the maintenance of spontaneous tone, LES smooth muscle contraction is mediated via a PKC-dependent pathway. When cytosolic calcium reaches a level sufficient to activate calmodulin, as may be achieved with a maximally effective concentration of ACh, LES smooth muscle contraction is mediated by a calmodulin-dependent pathway (Biancani et al., 1994) and the PKC-dependent pathway is inhibited (Biancani et al., 1994; Chakravarthy et al., 1995a, b; Kraft and Anderson, 1983; Yu et al., 1993; Zhao et al., 1991). The mechanism of calmodulin-induced inhibition of PKC activity has not been investigated extensively. Kruger et al. (1990) examined tryptic fragments of calmodulin and found that two PKC-inhibitory sequences where localized to the second and fourth calcium binding domains of calmodulin and that calmodulin-induced PKC inhibition was not affected by calmodulin antagonists.

Because AE reduces endogenous resting 1,4,5-IP_3 levels (Biancani et al., 1992) and damages intracellular calcium stores in the LES (Rich et al., 1997), we hypothesized that maximal ACh-induced contraction may be mediated by a PKC-dependent pathway after the induction of AE. We found that after the induction of AE, ACh-induced contraction was inhibited by the PKC antagonist H7 but not by the calmodulin antagonist CGS9343B, and these changes were mimicked in normal cells by acutely depleting calcium stores by exposure to thapsigargin (Rich et al., 1997). Thapsigargin causes release of intracellular Ca^{2+} and inhibits ATP-dependent Ca^{2+} uptake, which results in depletion of intracellular Ca^{2+} stores. These data support the view that changes in signal transduction pathways may be related to the Ca^{2+} levels available to support contraction. In the present investigation, we therefore examined in some detail the characteristics of the pathway mediating contraction in response to a maximally effective dose of ACh in AE. Because AE causes a switch from an 1,4,5-IP_3- and calmodulin-dependent to a PKC-dependent pathway, we measured PKC activity and 1,4,5-IP_3 production in response to AE stimulation in normal and AE LES muscle.

To test the functional significance of PKC in normal and AE LES, we examined PKC translocation from cytosol to membrane in response to ACh stimulation of intact muscle strips. Activation of PKC is associated with the translocation of the enzyme from the cytosol to the membrane fraction (Khalil and Morgan, 1991; Kraft and Anderson, 1983; Nishizuka, 1986). The dose of ACh (10^{-5} M) used was previously shown to produce a maximal contraction in LES smooth muscle strips (Biancani et al., 1987) and is different from the maximally effective concentration of ACh used in studies of isolated smooth muscle cells.

We found that in normal LES muscle tissue ACh-induced PKC activation was maximal at an intermediate ACh concentration and that a maximally effective dose of ACh, which causes contraction through a calmodulin-dependent pathway, causes little increase in PKC activity when compared with unstimulated muscle. In contrast, after induction of AE, PKC activity increased dose-dependently with increasing ACh concentrations and was 3-fold greater than control values in response to maximal ACh. These results are consistent with calmodulin-induced inhibition of PKC.

To determine whether 1,4,5-IP_3 plays a role in ACh-induced contraction in AE, we examined formation of inositol phosphates, in response to a maximally effective dose of ACh.
Rapid breakdown of phosphatidylinositol 4,5-bisphosphate by PI-PLC results in the formation of 1,4,5-IP₃, which is metabolized to 1,4-IP₂ and 1-IP₁. 1,4-IP₂ and 1,4,5-IP₃ are also produced by PI-PLC-induced hydrolysis of phosphatidylinositol 4-phosphate (Berridge, 1987; Berridge and Irvine, 1984; Michell, 1975; Murthy and Makhlof, 1991, 1995). In our system, as reported previously (Sohn et al., 1993), 1-IP₁, 1,4-IP₂ and 1,4,5-IP₃ were increased by ACh stimulation in normal LES muscle at 1 and 5 s after administration of ACh, i.e., in a time frame consistent with contraction induced by ACh (Hillemeier et al., 1991). In contrast, after AE none of the inositol phosphates were increased by ACh stimulation in LES muscle. These data support the view that 1,4,5-IP₃ plays a role in ACh-induced contraction of LES muscle in normal but not in AE animals. Because 1,4,5-IP₃-induced calcium release is thought to activate a calmodulin-myosin light chain kinase pathway these data also support our previous findings that contraction of normal LES, but not of AE LES, is affected by the calmodulin antagonist CGS9343B (Rich et al., 1997).

Because AE causes a switch from an 1,4,5-IP₃- and calmodulin-dependent to a PKC-dependent pathway we examined the transduction mechanisms in LES smooth muscle cells. We found that AE produced a change in the muscarinic receptors, G-proteins and phospholipases activated by stimulation with a maximally effective dose of ACh.

**Different muscarinic receptors mediate ACh-induced contraction in normal LES cells and AE cells.** The pharmacologic classification of muscarinic receptors is based on different receptor affinities for selective antagonists. In the present investigation we examined the effect of pirenzepine, methoctramine and pF-HSD on ACh-induced contraction. Pirenzepine is a tricyclic drug with higher selectivity for M₁ relative to M₂ or M₃ muscarinic receptors (Caulfield, 1993; Dorje et al., 1991). Methoctramine is the prototype of the polymethylene tetramine class of muscarinic receptor antagonists which have been reported to display selectivity toward cardiac M₂ muscarinic receptors (Giraldo et al., 1988; Melchiorre, 1988; Melchiorre et al., 1987a, b). pF-HSD is an hexahydro-sila-difenidol analog which has been shown to possess considerable selectivity for the smooth muscle M₃ muscarinic receptor in guinea pig ileum (Lambrecht et al., 1988, 1989).

We have previously reported that ACh-induced contraction of normal LES smooth muscle cells was significantly inhibited by the M₃ muscarinic antagonist pF-HSD (Sohn et al., 1993).

In the present study we show (table 1) that inhibition of contraction by pirenzepine in normal and AE LES, and by methoctramine in normal LES were relatively low and not different form each other. This inhibition may be related to nonselective binding of antagonists to muscarinic receptors.

Methoctramine-induced inhibition was significantly greater in AE than in normal LES (unpaired t test, P < .05), and by the M₃ antagonist pF-HSD (ANOVA, ""P < .001), which suggests that ACh activates both M₂ and M₃ receptors. (B) The response to maximal ACh was mediated by both G₉/₁₁ and G₁₃, as in AE LES cells, because ACh-induced contraction was significantly antagonized by antibodies against G₉/₁₁ and G₁₃ (ANOVA, ""P < .001). (C) In addition, ACh-induced contraction was reduced by the PI-PLC antagonist U73122, the PC-PLC inhibitor D609 and the PLD pathway inhibitor propranolol (ANOVA, ""P < .001).

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**Fig. 6.** Depletion of intracellular calcium stores in normal LES cells reproduces the changes in signal transduction induced by AE. After 30 min incubation of normal LES circular smooth muscle cells in 10⁻⁶ M thapsigargin to deplete intracellular Ca²⁺ stores, (A) the response to maximal ACh was significantly inhibited by the M₂ antagonist methoc...
contraction is mediated not only through M₃, as in the normal LES, but also by M₂ receptors.

**Different G-proteins mediate ACh-induced contraction in normal LES and AE cells.** G-proteins transduce ligand binding to a cell surface receptor into intracellular signals. Antibodies, raised against synthetic peptides corresponding to the amino acid sequence of the COOH-terminal of specific G-protein α subunits, have been used as effective probes of G-protein structure and function (Goldsmith et al., 1988; Gutowski et al., 1991; Shenker et al., 1991; Simonds et al., 1989; Sohn et al., 1993; Spiegel et al., 1990). We used these antibodies to identify the G-proteins coupled to the muscarinic receptors in LES circular muscle.

We have previously demonstrated the presence of G₉/₁₁, G₁₃ and G₁₁₋₂ type G-proteins in normal LES smooth muscle cells by Western blot (Sohn et al., 1995). In normal LES smooth muscle only G₉/₁₁ antibodies inhibited G-proteins of the G₉/₁₁ type (Sohn et al., 1993). After induction of AE, ACh-induced contraction was inhibited by both M₂ and M₃ receptors and by antibodies raised against both the G₉/₁₁- and the G₁₃-type G-protein. This suggests that in addition to a M₂-G₉/₁₁ coupled pathway, a second M₂-G₁₃-dependent pathway is activated after induction of AE.

**Different phospholipases mediate ACh-induced contraction in normal LES and AE cells.** We have previously shown that in normal LES, contraction in response to a maximally effective dose of ACh results from PI-PLC activation, because it is associated with an increase in inositol phosphates and is inhibited by the PI-PLC inhibitor U-73122 (10⁻⁶ M) (Biancani et al., 1994; Sohn et al., 1993). U-73122 is an amphipathic cation which reversibly competes with calcium for the binding site on PLC that regulates expression of the phospholipase activity (Bleasdale et al., 1989), and is capable of reducing spontaneously elevated 1,4,5-IP₃ levels in LES circular muscle (Biancani et al., 1994) without affecting contraction of normal esophageal smooth muscle cells, which is PKC-dependent and mediated by PC-PLC, PLD and PLA₂ (Sohn et al., 1993, 1994a, b, 1995). In the current study, we show that after induction of AE, LES contraction is inhibited not only by U73122, but also by D609 and propranolol, which suggests that other phospholipases mediate ACh-induced contraction of LES besides PI-PLC.

D609 is thought to be a selective inhibitor of PC-PLC (Schutze et al., 1992). Propranolol at high concentrations is thought to inhibit PLD-mediated production of DAG from phosphatidylcholine (Billah and Anthes, 1990; Exton, 1990; Lassegue et al., 1991; Welsh et al., 1990). Phosphatidylcholine hydrolysis by PC-specific PLC and PLD has been shown to be an alternative source of DAG (Billah and Anthes, 1990; Exton, 1990; Lassegue et al., 1991; Welsh et al., 1990). PC-specific PLC produces DAG and phosphocholine, whereas PC-specific PLD produces choline and phosphatidic acid, which is metabolized to DAG by phosphatidic acid phosphohydrolase (Billah and Anthes, 1990; Dennis et al., 1991). D609 blocks PC-specific PLC activity derived from Bacillus cereus, without affecting PLA₂, PLD and PI₃-specific PLC activity (Schutze et al., 1992). High concentrations (0.1–1 mM) of propranolol have been shown to reduce DAG production by inhibition of phosphatidic acid phosphohydrolase without affecting phosphatidylcholine-specific PLD activity.
or PI-PLC activity (Billah et al., 1989; Qian and Drewes, 1990, 1991).

ACh-induced contraction of LES after the induction of AE was antagonized by D609 and by the phosphatidic acid phosphohydrolase inhibitor propranolol, which had no effect on contraction of normal LES muscle. This finding is consistent with the view that after the induction of AE LES contraction may be mediated by activation of PC-specific PLC and PLD, resulting in hydrolysis of PC rather than of PI.

We conclude that after induction of AE, contraction induced by a maximally effective dose of ACh is mediated by M₂ and M₃ receptors, activating G₁₃, G₁₁, and the phospholipases PI-PLC, PC-PLC and PLD.

M₂ receptors are linked to G₁₁ and PI-PLC; M₂ receptors are linked to G₁₃ and PI-PLC and PLD. To test whether the multiple muscarinic receptors, G-proteins and phospholipases are organized in distinct pathways, we examined the effect of muscarinic antagonists and phospholipase inhibitors in the presence of a maximally effective dose of selective G-protein antibodies. Permeabilized smooth cells from esophagitis cats were incubated in either G₁₁ or G₁₃ antibodies, then exposed to muscarinic or phospholipase antagonists before ACh administration. The antibodies were used at a maximally effective concentration to maximally reduce the pathway activated by a specific G-protein, before using additional inhibitors. When the pathway blocked by a selective G-protein antibody is maximally inhibited, addition of muscarinic receptor antagonists or phospholipase inhibitors can cause additional inhibition only when linked to a pathway different from the one inhibited by the G-protein antibody.

We found that, in the presence of G₁₁ antibodies, no additional inhibition was produced by either pF-HSD or by U73122, which suggests that G₁₁-type G-proteins participated in the pathway containing M₂ muscarinic receptors and PI-PLC. In contrast, metochnoramine, propranolol and D609 caused additional and almost complete inhibition in the presence of G₁₁ antibodies, which suggests that the pathway inhibited by these agents was not coupled G₁₁.

Conversely, in the presence of G₁₃ antibodies, no additional inhibition was produced by either metochnoramine, propranolol and D609, which suggests that G₁₃-type G-proteins participated in the pathway containing M₂ muscarinic receptors, PC-PLC and PLD. In contrast, pF-HSD and U73122 caused additional and almost complete inhibition in the presence of G₁₃ antibodies, which suggests that the pathway inhibited by these agents was not coupled G₁₃.

The results of the current study demonstrate that, in contrast to the normal LES in which ACh, at a maximally effective dose, activates only M₂, G₁₁ and PI-PLC, after induction of esophagitis, a second pathway is activated. This pathway, which is similar to the one that has been previously reported for the esophageal body (Sohn et al., 1993), consists of M₂ muscarinic receptors linked to G₁₃-type G-proteins and, PC-PLC and PLD-type phospholipases.

Depletion of intracellular calcium stores in normal LES cells mimics changes induced by AE. We have previously reported that AE reduces resting 1,4,5-IP₃ levels and intracellular Ca⁺⁺ stores (Rich et al., 1997). In the current study, we demonstrate that a reduction in the intracellular calcium stores of normal LES smooth muscle cells by 30 min incubation in thapsigargin is sufficient to reproduce the changes in signal transduction observed in AE. We show that ACh-induced contraction of thapsigargin-treated normal LES cells is mediated by M₂ and M₃ receptors, activating G₁₁, G₁₃ and the phospholipases PI-PLC, PC-PLC and PLD and these muscarinic receptors, G-proteins and phospholipases are organized in distinct pathways. After pharmacologic depletion of intracellular Ca⁺⁺ stores with thapsigargin, ACh-induced contraction of LES smooth muscle cells is mediated not only by M₃ muscarinic receptors linked to G₁₁-type G-proteins and PI-PLC, but also by M₂ muscarinic receptors linked to G₁₃-type G-proteins and PC-PLC and PLD-type phospholipases, as occurs after induction of AE.

These results support the hypothesis that ultimately the amount of Ca⁺⁺ available for release from intracellular stores of LES smooth muscle defines the signal transduction pathway activated by a maximally effective dose of ACh, beginning with events localized to the membrane such as activation of particulate muscarinic receptors, G-proteins and phospholipases down to activation of such effectors as calmodulin and PKC. These data suggest that a pathway activated by G₁₁-G₁₃, resulting in production of DAG and activation of PKC, may be present in the normal LES, even though its contribution is not apparent during contraction induced by a maximally effective dose of ACh. In the normal LES a maximally effective dose of ACh may cause Ca⁺⁺ release of a magnitude sufficient to activate calmodulin and eliminate the contribution of the PKC-dependent pathway, resulting in calmodulin-dependent, PKC-independent contraction. After depletion of intracellular Ca⁺⁺ stores by thapsigargin (or acute esophagitis) Ca⁺⁺ release may be drastically reduced, and the available Ca⁺⁺ may be insufficient to activate calmodulin, and inhibit PKC activity. Thus the contribution of a PKC-dependent pathway is “unmasked” by activation of M₂ receptors, G₁₃ G-proteins and DAG production. Contraction becomes calmodulin-independent and mediated through a PKC-dependent pathway.

In addition to reduced resting 1,4,5-IP₃ levels in AE LES, in the current study we show that there is no measurable production of 1,4,5-IP₃ in response to maximal ACh stimulation, suggesting that esophagitis may cause damage to PI-PLC. However, the PI-PLC-selective antagonist U73122 inhibited contraction in response to maximal ACh stimulation, suggesting that PI-PLC plays a role in LES contraction, presumably through hydrolysis of PIP₂ and production of 1,4,5-IP₃ and DAG.

To reconcile these apparently contradictory findings, we considered the possibility that U73122 may not be selective for PI-PLC. However, we previously reported that U73122 significantly inhibited tone and contraction in response to a maximally effective dose of ACh in normal LES without affecting contraction in normal esophageal smooth muscle cells, which is PKC-dependent and mediated by PC-PLC, PLD and PLA₂ (Sohn et al., 1993, 1994a, b, 1995). In addition, U73122 inhibited spontaneous tone and 1,4,5-IP₃ formation in normal LES muscle (Biancani et al., 1994; Hillemeier et al., 1996). These findings demonstrate that in our system U73177 is selective enough to inhibit PI-PLC in LES muscle without affecting PC-PLC, PLD or PLA₂ in the esophageal muscle. In the present study, we show that inhibiting G₁₁, which is known to be linked to PI-PLC, has the same effect as inhibiting PI-PLC, and the antibodies used to inhibit G₁₁ are reasonably selective (Sohn et al., 1995; Sohn
et al., 1993). Therefore, the fact that inhibition of PI-PLC by U73122, and inhibition of G_{q/11} by the antibodies significantly reduces LES contraction in response to ACh, suggests that PI-PLC contributes to ACh-induced contraction.

An alternative hypothesis that may be considered is that the inositol phospholipids, which are the substrates for PI-PLC and constitute only a small percent of membrane phospholipids (Billah and Anthes, 1990), may be depleted as a consequence of AE. Numerous phospholipids are present in cell membranes, and in the absence of inositol phospholipids, it is possible that other substrates may be hydrolyzed by one or more of the almost 20 PI-PLC isozymes discovered to date. This hypothesis is not entirely consistent with previous characterizations of PI-PLCs, however, which have been reasonably selectively for inositol phospholipids (Exton, 1996; Lee and Rhein, 1995; Späth et al., 1991). We can only speculate that there may be a U73122-sensitive phospholipase, which in some circumstances may hydrolyze non-inositol-containing membrane phospholipids.

We conclude that in normal LES cells contraction is calmodulin-dependent and mediated by M₃ receptors linked to G_{q/11} and PI-PLC, whereas in AE, contraction through this pathway is reduced because 1,4,5-IP₃ production and Ca^{2+} stores are damaged. In AE a second PKC-dependent pathway is activated, which is mediated by M₂ receptors linked to G_{q/11}, PC-PLC and PDL.

References


affects intracellular calcium stores in the cat lower esophageal sphincter.


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