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Cooperation of Gβ and Gαq Protein in Contractile Response of Cat Lower Esophageal Sphincter (LES)

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We previously shown that LES contraction depends on M_3 receptors linked to PTX insensitive G_q protein and activation of PLC. This results in production of IP_3, which mediates calcium release, and contraction through a CaM dependent pathway. In the esophagus ACh activates M_2 receptors linked to PTX sensitive G_{i3} protein, resulting in activation of PLD, presumably, production of DAG. We investigated the role of PLC isozymes which can be activated by Gq or Gβ protein on ACh-induced contraction in LES and esophagus. Immunoblot analysis showed the presence of 3 types of PLC isozymes, PLC-β1, PLC-β3, and PLC-γ1, but not PLC-β2, PLC-β4, PLC-γ2, PLC-δ1, and PLC-δ2 from both LES and esophageal muscle. ACh produced contraction in a dose dependent manner in LES and esophageal muscle cells obtained by enzymatic digestion with collagenase. PLC-β1 or PLC-β3 antibody incubation reduced contraction in response to ACh in LES but not in esophageal permeabilized cells, but PLC-γ1 antibody incubation did not have an inhibitory effect. The inhibition by PLC-β1 or PLC-β3 antibody on Ach-induced contraction was antibody concentration dependent. The combination with PLC-β_1 and PLC-β_3 antibody completely abolished the contraction, suggesting that PLC-β1 and PLC-β3 have a synergism to inhibit the contraction in LES. PLC-β1, -β3 or -γ1 antibody did not reduce the contraction of LES cells in response to DAG (10^{-6} M), suggesting that this isozyme of PLC may not activate PKC. When $G_{q/11}$ antibody was incubated, the inhibitory effect of the incubation of PLC β_3, but not of PLC β_1 was additive (Fig. 6). In contrast, when G_β antibody was incubated, the inhibitory effect of the incubation of PLC β_1, but not of PLC β_3 was additive. This data suggest that $G_{q/11}$ or Gβ may activate cooperatively different PLC isozyme, PLCβ_1 or PLCβ_3 respectively.

Key Words: Gβ, Gα_q, Phospholipase C, LES, Contraction

INTRODUCTION

The LES is characterized by the ability to maintain a sustained pressure, and to relax allowing the passage of a bolus, whereas the esophagus normally relaxes and contracts only briefly, when required to produce peristalsis. The neuromuscular mechanisms which participate in the physiological regulation of these functions are not well understood, but it is thought that LES tone is spontaneous and regulated mostly through myogenic mechanisms, whereas LES relaxation and esophageal contraction are induced by neural mechanisms.

Esophageal reflux is a common condition that affects children and one in ten adults and, if untreated, may result in chronic esophagitis, aspiration pneumonia, esophageal strictures, and Barretts esophagus, a premalignant condition. Esophagitis is a multifactorial disease that may depend on inappropriate LES relaxation, speed of esophageal clearance, mucosal resistance and other factors, and is often associated with low LES pressure (Dodds et al, 1982; Helms et al, 1983, 1984).

We found that intracellular pathways utilized by LES muscle to maintain tone and to contract in response to ACh, are entirely different from those utilized by esophageal muscle to contract in response to ACh, which is at least in part responsible for muscle contraction during esophageal peristalsis. In smooth muscle cells isolated by enzymatic digestion from the circular layer of LES, contraction in response to a maximally effective dose of ACh is mediated through IP_3 induced Ca^{2+} release from intracellular stores, and activation of a calmodulin- dependent pathway (Biancani et al, 1994).

In esophageal circular muscle, however, ACh-induced contraction mediated through IP_3 and calmodulin-independent and mediated through a PKC-dependent pathway (Sohn et al, 1993, 1994), involving a calcium insensitive PKCε (Sohn et al, 1997). In this pathway, calcium is required for activation of phospholipases and production of the second messengers DAG and arachidonic acid (AA) (Sohn et al, 1994). When the second messengers are present, activation of PKCε and contraction can proceed even in the absence of intracellular calcium and is not affected by calmodulin and MLC Kinase antagonists. The precise mechanisms responsible for mediation of this

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ABBREVIATIONS: LES, Lower esophageal sphincter; PTX, Pertussis toxin; PLC, Phospholipase C; DAG, Diacylglycerol; AA, Arachidonic acid; MLC, Myosin light chain; PKC, Protein kinase C.

PKC-dependent contraction are not well established (Bitar et al, 1991; Khalil & Morgan, 1992, 1993; Allen & Walsh, 1994; Mennice et al, 1997), but since calcium and calmodulin are not required to support the PKC ε-mediated contraction of esophageal muscle, we have proposed that a Ca^{2+} -calmodulin-MLC Kinase-dependent pathway may not be involved in the regulation of this type of contraction (Sohn et al, 2000).

Three families of mammalian PLC isozymes, PLC-β, -γ, and -δ, have been described based on their molecular structure and mechanism of regulation (Rhee & Bae, 1997), and to be activated by Gα and G$\beta\gamma$ subunits of the heterotrimeric G proteins, while PLC-γ isozymes are regulated by tyrosine phosphorylation (Rhee & Bae, 1997). PLC-δ isozymes are smaller (85 kDa) than PLC-β and -γ (150 and 145 kDa, respectively) and their function remains unclear (Rhee & Bae, 1997). The α subunits of these proteins activate phospholipase C-β isoenzymes (Smrcka et al, 1991), but not phospholipases (PLCs) Cγ or Cδ (Lee et al, 1994). PLC-β isozymes may be stimulated by both G-proteins α subunits of the G_q family and by free $\beta\gamma$ subunits (Camps et al, 1992).

Our aim was to investigate the role of PLC isozymes activated by $G_{q/11}$ or Gβ protein on ACh-induced contraction in cat LES and esophageal circular muscle cells.

METHODS

Drugs and chemicals

PLC-β antibodies (PLC-β_1, PLC-β_2, PLC-β_3, PLC-β_4, PLC-γ_1, PLC-γ_2, PLC-δ_1, and PLC-$\delta_{2)}$, Gq and Gβ antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); L-alpha-1,2-dioctanoylglycerol (DAG) from Avanti Polar Lipids (Pelham, AL, USA); chemiluminescence agents from NEN Life Science (Bonston, MA, USA); PBS from Boehringer Mannheim (Indianapolis, IN, USA); collagenase from Worthington Biochemiclas (Freehold, NJ, USA); rainbow prestained-molecular weight marker from Amersham (Arlington Heights, IL, USA); SDS sample buffer and nitrocellulose membrane from BioRad (Richmond, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit antibody were obtained from Pierce (Rockford, IL, USA); acetylcholine, saponin, EGTA, EDTA, HEPES, soybean trypsin inhibitor, BSA, Ponseau S, leupeptin, aprotinin, and β-mercaptoethanol from Sigma (St. Louis, MO); All other chemicals were of the highest purity or molecular biology grade available from commercial sources.

LES and esophageal muscle layer harvest

Adult cats of either sex weighing between 3 and 5 kg were euthanized, and LES smooth muscle layer was obtained as previously described (Biancani et al, 1987). Briefly, the chest and abdomen were opened with a mid-line incision exposing the esophagus and stomach. The esophagus and stomach were removed together and pinned on a wax block at their in vivo dimensions and orientation. The esophagus and stomach were opened along the lesser curvature. After opening the esophagus and stomach and identifying the LES, the mucosa and submucosal connective tissue were removed by sharp dissection. The LES was excised and a 3~5 mm wide strip at the junction of LES and esophagus was discarded to avoid overlap. The circular muscle layer from the LES and esophagus was cut into 0.5 mm thick slices with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA). The last slices containing the myenteric plexus, longitudinal muscle and serosa were discarded.

Dispersion of LES muscle cells

Tissue squares got from chopping with hand were digested in HEPES buffer, containing 0.1% collagenase type II for two hours to get isolated smooth muscle cells, as previously described (Biancani et al, 1987). The HEPES solution contained: NaCl 115 mM, KCl 5.8 mM, KH_2PO_4 12 mM, glucose 2.5 mM, (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) sodium salt (HEPES) 25 mM, $CaCl_2$ 2 mM, $MgCl_2$ 0.6 mM, BME aminoacid supplement 0.3 mg/ml, soybean trypsin inhibitor (0.09 mg/ml). The solution was gently gassed with 100% O_2. At the end of the digestion period, the tissue was poured out over a 450 m nylon mesh (Tetko Inc., Elmsford, NY), rinsed in collagenase-free HEPES buffer to remove any trace of collagenase, and then incubated in this solution at 31°C, gassed with 100% O_2. The cells were allowed to dissociate freely for 10~20 minutes.

Preparation of permeabilized smooth muscle cells

Cells were permeabilized, when required to allow the use of agents such as PLC antibodies and G protein antibodies which do not diffuse across the intact cell membrane. When PLC antibodies and G protein antibodies were used, permeabilized cells were incubated with the antibody at a 1/200 dilution for 60 min prior to addition of ACh or DAG. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with an enzyme-free cytosolic buffer of the following composition (mM): NaCl 20, KCl 100, $MgSO_4$ 5.0, NaH_2PO_4 0.96, EGTA 1.0, and $CaCl_2$ 0.48 and 2 % bovine serum albumin. The cytosolic buffer was equilibrated with 95% O_2 5% CO_2 to maintain a pH of 7.2 at 31°C. Muscle cells dispersed spontaneously in this medium. The cytosolic buffer contained 0.48 mM $CaCl_2$ and 1 mM EGTA, yielding 0.18 M free Ca^{2+}, as calculated according to Fabiato and Fabiato (1979). After dispersion the cells were permeabilized by incubation for 3 min in cytosolic buffer containing saponin (75 g/ml). After exposure to saponin the cell suspension was spun at low g and the resulting pellet was resuspended in saponin-free modified cytosolic buffer containing antimycin A (10 M), ATP (1.5 mM), and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase (10 units/ml). After the cells were washed free of saponin they were resuspended in modified cytosolic buffer.

Agonist-induced contraction of isolated muscle cells

The cells of esophagus and LES were contracted by exposure for 30 sec to ACh. After exposure to agonists the cells were fixed in acrolein at a final 0.6% concentration. A drop of the cell-containing medium was placed on a glass slide, covered by a cover slip, and the edges were sealed with nail enamel to prevent evaporation.

The length of thirty consecutive intact cells encountered at random in each slide was measured with a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany),

and a closed-circuit video camera (model WV-CD51, Panasonic, Seacaucus, NJ) connected to a Macintosh Computer (Apple Inc., Cupertino, CA) with an image analysis software program (Image 1.56, National Institute of Health, Bethesda, MD). Contraction was expressed as % shortening of average of 30 consecutive cells compared with control.

Immunoblot analysis

LES and esophageal muscles were homogenized in a homogenizing buffer containing 20 mM tris (hydroxymethyl) aminomethane (Tris) HCl (pH 7.5), 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM ethylenediamine tetraacetic acid (EDTA), 10 μg/ml leupeptin, 10 μg/ml aprotinin 10 mM, and β-mercaptoethanol and centrifuged at 14,000 g for 15 min (G proteins) or 13,000 g for 5 min at 4°C. The supernatant was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (PLC-5%, G proteins-12.5% gradient gel). Prestained molecular mass markers were run in an adjacent lane to permit molecular mass determination. The separated proteins were transferred to nitrocellulose membranes in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol using power supply, Power Pac 1,000 (Bio-Rad, Melville, NY). The blots were incubated for 1 h at room temperature in PBS in 5% non-fat dry milk to block nonspecific antibody binding. After three or four washes in PBS, the blots were incubated for 1 hr at 4°C with isozyme-specific monoclonal antibodies to PLC isozymes (PLC-β_1, PLC-β_2, PLC-β_3, PLC-β_4, PLC-γ_1, γ_2, PLC-δ_1, and PLC-δ_2), and to G proteins (Gq and G_β). Antibody were removed by washing in PBS containing 0.05% Tween 20, followed by washing three times in PBS. This was followed by a 60-min incubation in horseradish peroxidase-conjugated goat anti-rabbit antibody (1 μg/ml). Detection was achieved with an enhanced chemiluminescence agent.

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 (Bio Rad Protein Assay; Bio Rad Laboratories, Richmond CA) according to the method of Bradford (1976).

Data analysis

Data are expressed as the mean±S.E.M. Statistical differences between means were determined by the Student's *t*-test. Differences between multiple groups were tested using analysis of variance (ANOVA) for repeated measures and checked for significance using the Scheffe F-test.

RESULTS

We previously shown that LES contraction depends on M_3 receptors linked to PTX insensitive G_q protein and activation of PLC. This results in production of IP_3, which mediates calcium release, and contraction through a CaM dependent pathway. In the esophagus ACh activates M_2 receptors linked to PTX sensitive G_{i3} protein, resulting in activation of PLD, and, presumably, production of DAG. It is possible that Gq may be couled to a certain subtype of PLC.

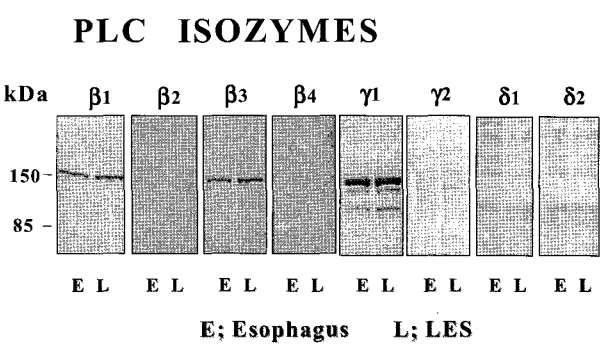

Fig. 1. Identification of PLC by Western blot. Solubilized tissue samples were loaded into SDS polyacrylamide gel electrophoresis system, transferred to NC membrane and incubated each PLC antibody at a 1 : 500 concentration for 60 min with shaking. This analysis showed the presence of the main PLC isozyme, PLC-β1 (150 kDa), PLC-β3 (150 kDa), and PLC-γ1 (145 kDa), but not PLC-β2 (150 kDa), PLC-β4 (150 kDa), PLC-γ2 (145 kDa), PLC-δ1 (85 kDa), and PLC-δ2 (85 kDa) from LES (L) and esophageal muscle (E).

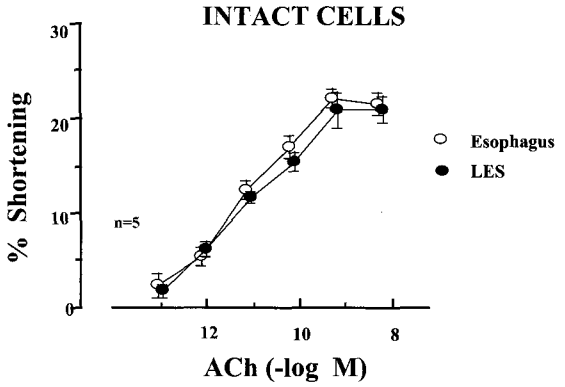

Fig. 2. Dose response curve of ACh-induced contraction of circular muscle cells of the LES and esophagus. Intact cells were contracted with the indicated concentration of ACh each dose. The values are the means±S.E. of five animals with 30 cells counted at random for each data point.

To do this, it was identified PLC isozymes using PLC isozyme antibody in immunoblot analysis (Fig. 1). This analysis showed the presence of the main PLC isozyme, PLC-β1 (150 kDa), PLC-β3 (150 kDa), and PLC-γ1 (145 kDa), but not PLC-β2 (150 kDa), PLC-β4 (150 kDa), PLC-γ2 (145 kDa), PLC-δ1 (85 kDa), and PLC-δ2 (85 kDa) from LES and esophageal muscle. ACh produced contraction in a dose dependent manner in LES and esophageal muscle cells cells which is obtained by enzymatic digestion with collagenase (Fig. 2). PLC-β1 or PLC-β3 antibody (1 : 100 dilution) incubation reduced by 50~57% contraction in response to ACh in LES but not in esophageal permeabilized cells (Fig. 3, $P<0.05$ by ANOVA), but PLC-γ1 antibody incubation did not have an inhibitory effect. The inhibition by PLC-β1 or PLC-β3 antibody on ACh-induced contraction was diluted concentration (1 : 200, 500, 1000) dependent (Fig. 4, $P<0.05$ or $P<0.01$ by ANOVA). The combination with PLC-β_1 and PLC-β_3 antibody (1 : 200

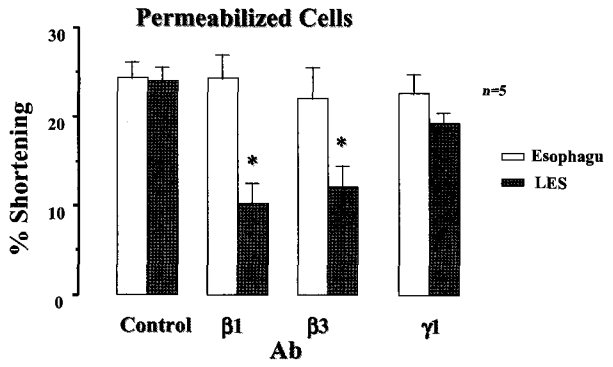

Fig. 3. Contraction is mediaited by PLC $\beta 1$ or $\beta 3$ in LES but not in esophagus. Muscle cells were permeabilized with saponin and preincubated for 60 min in cytosolic medium containing PLC Antibody (1:00) to allow diffusion of the antibody into the cytosolic region of the cell membrane. PLC-$\beta 1$ or PLC-$\beta 3$ antibody partially reduced by the contraction in response to ACh in LES but not in esophageal permeabilized cells. The values are the means ± S.E. of five animals with 30 cells counted at random for each data point. *P < 0.05 by ANOVA.

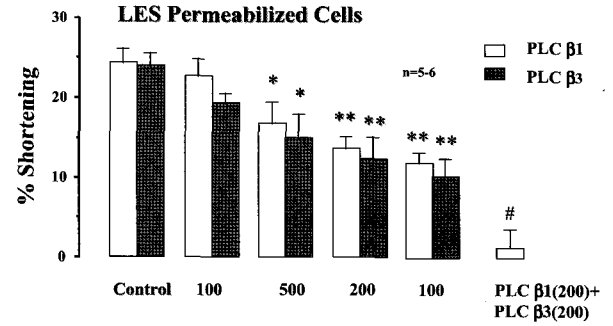

Fig. 4. The cooperation of PLC $\beta 1$ and $\beta 3$ on the inhibitory effect in LES muscle cells. The inhibition by PLC-$\beta 1$ or PLC-$\beta 3$ antibody on ACh-induced contraction was antibody dilution dependent. The combination with PLC-$\beta 1$ and PLC-$\beta 3$ antibody (1 : 100 dilution) abolished the contraction (# P < 0.05 by t-test). The values are the means ± S.E. of five-six animals with 30 cells counted at random for each data point. *P < 0.05 or **P < 0.01 by ANOVA to the control.

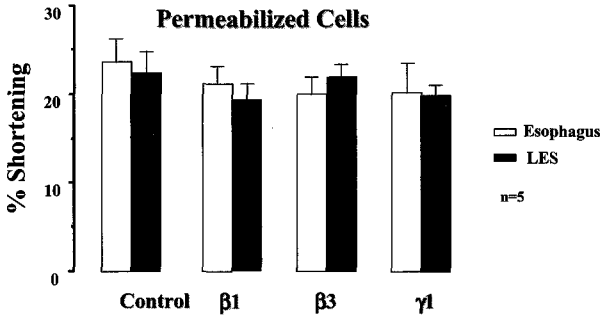

Fig. 5. PLC isozymes on DAG (10^{-7} M for esophagus and 10^{-6} M for LES)-induced contraction in permeabilized cells. The values are the means ± S.E. of five animals with 30 cells counted at random for each data point.

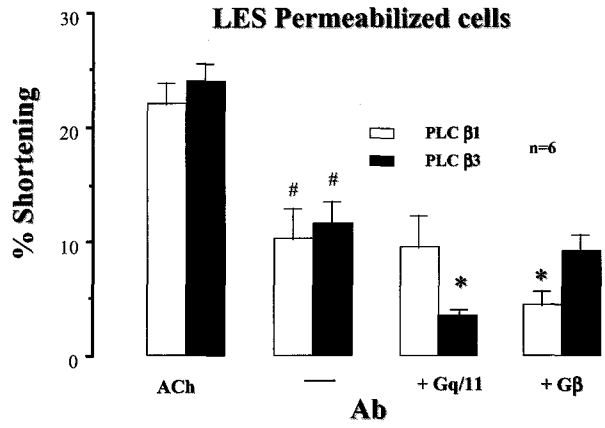

Fig. 6. The differential activation of G protein in mediating PLC $\beta 1$ or $\beta 3$-induced contraction. ACh-induced contraction was partially reduced by PLC $\beta 1$ or PLC $\beta 2$ antibody (# P < 0.05 by t-test). When Gq/11 antibody was incubated, the inhibitory effect of the incubation of PLC $\beta 3$ but not of PLC β_1 was additive (*P < 0.05 by ANOVA). In contrast, when Gβ antibody was incubated, the inhibitory effect of the incubation of PLC β_1 but not of PLC $\beta 3$ was additive (*P < 0.05 by ANOVA). These data suggest that Gq/11 or Gβ may activate different PLC isozyme (PLC$\beta 1$ or PLC$\beta 3$).

dilution) completely abolished the contraction, suggesting that PLC-$\beta 1$ and PLC-$\beta 3$ have a synergism to inhibit ACh-induced contraction in LES (P < 0.05).

Furthermore we could try in LES and esophagus cells that PLC isozyme (β_1, β_3, or $\gamma 1$) may activate PKC dependent pathway (Fig. 5). The cells were contracted by exposure for 30 sec to, PKC an agonist, DAG (10^{-7} M for esophagus and 10^{-6} M for LES; maximum contraction already reported) in permeabilized cells. These concentrations produce a maximal contraction of the circular smooth muscle from these two distinct tissue types. However, these antibodies (PLC-β_1, PLC-β_3, or PLC-$\gamma 1$) did not reduce the contraction of permeabilized LES cells in response to DAG (10^{-6} M), suggesting that this isozyme of PLC in LES may not activate PKC.

We already have shown that G_q (42 kDa), G_{i1} (40 kDa), G_{i2} (40 kDa), G_{i3} (40 kDa), G_o (40 kDa), G_s (46 kDa) were detected and the activation of G_q coupled to PLC contribute ACh-induced contraction (Sohn et al, 1995). These data are similar to those of bladder (Yang et al, 2000). We wonder which PLC isozyme may be activated by a G protein subunit (Gq or/and Gβ). ACh-induced contraction was partially reduced by PLC $\beta 1$ or PLC $\beta 2$ antibody (Fig. 6, P < 0.05 by t-test). When $G_{q/11}$ antibody was incubated, the inhibitory effect of the incubation of PLC β_3, but not of PLC β_1 was additive (Fig. 6, P < 0.05 by ANOVA). In contrast, when G_β antibody was incubated, the inhibitory effect of the incubation of PLC β_1, but not of PLC β_3 was additive (P < 0.05 by ANOVA). This data suggest that $G_{q/11}$ or Gβ may activate different PLC isozyme (PLCβ_1 or PLC β_3).

PLC isozymes (PLC β_1 and PLCβ_3) mediated ACh-induced contraction of LES but not of esophagus, and PLC β_1 and PLCβ_3 -induced cooperation was activated by Gq and Gβ protein, respectively.

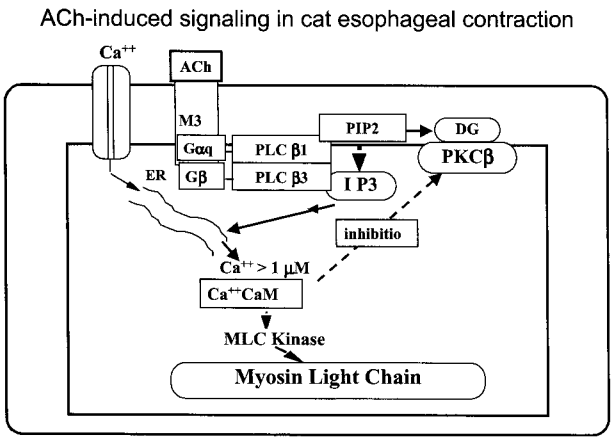

Fig. 7. The role of PLC isozymes activated by $G_{q/11}$ or $G\beta$ protein on ACh-induced contraction in cat LES. Contraction of LES cells by a maximally effective dose of ACh is mediated by activation of phosphatidylinositol-specific phospholipase C (PI-PLC), and production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes release of Ca^{2+} from stores at a concentration sufficient to cause activation of calmodulin (CAM). Ca^{2+}-CaM causes activation of myosin light chain kinase (MLC kinase) and inhibition of protein kinase C (PKC), inducing a contraction that is entirely calmodulin-dependent. Ca^{2+}-CaM-induced inhibition of PKC masks the presence of other factors that would otherwise contribute to activation of PKC. Activated CaM by high Ca^{2+} inhibit PKC-mediaed signaling. This data suggest that $G_{q/11}$ or $G\beta$ may activate cooperatively different PLC isozyme, PLCβ_1 or PLCβ_3 respectively.

DISCUSSION

G-proteins are highly homologous and consist of three subunits (α, β, γ) (Gilman, 1987; Spiegel, 1987; Lechleiter et al, 1990). Activation of a G-protein by an agonist-liganded receptor results in the exchange of GDP, bound to the a subunit, for GTP and dissociation of the α-GTP complex from the $\beta\gamma$ heterodimer. The α subunit with GTP bound and the free $\beta\gamma$ subunit may then interact with effector proteins such as ion channels or enzymes that generate second messengers such as cAMP and inositol 1, 4, 5-trisphosphate. Termination of the signal occurs when the GTP bound to the α subunit is hydrolyzed to GDP, and the α subunit and $\beta\gamma$ complex reassociate (Simon et al, 1991).

The α subunits of G-proteins contains the site(s) for NAD-dependent ADP-ribosylation by bacterial toxins. Members of the Gi class of α subunits contain sites susceptible to modification by PTX and may mediate activation of PTX-sensitive processes. This toxin uncouples the receptor from its G-protein and blocks signal transduction (Ui, 1990). The Gq class of α subunits lack the cysteine residue located four amino acids away from the COOH-terminal that is the target for PTX-mediated ADP-ribosylation. Therefore, previous data suggested that LES M_3 receptors may be linked to a G-protein of the Gq class while esophageal M_2 receptors may be coupled with a G-protein of the Gi class (Sohn et al, 1993).

In LES circular muscle cells isolated by enzymatic digestion, contraction in response to maximally effective doses of ACh or IP_3 depends on release of Ca^{2+} from intracellular stores and activation of a calmodulin- dependent pathway (Biancani et al, 1994). In contrast, maintenance of tone and response to low doses of ACh or IP_3 depend on a PKC-mediated pathway (Biancani et al, 1994; Hillemeier, 1996). In contrast, contraction of esophageal muscle in response to ACh depends on activation of phospholipases and production of DAG and AA, without any measurable production of IP_3 (Sohn et al, 1993, 1994, 1995). Once DAG and AA are produced, they interact to activate a Ca^{2+}-independent PKC ε (Sohn et al, 1994, 1997). This pathway does not involve calmodulin or myosin light chain kinase (Sohn et al, 2000). Esophagus muscle cells do not contract in response to exogenous calmodulin or MLC Kinase under the same conditions that cause maximal contraction of LES cells, and the calmodulin antagonist CGS9343B or the MLC kinase antagonists quercetin or ML7 do not inhibit ACh-induced contraction of ESO cells (Sohn et al, 2000).

Clearly the different modes of activation of these muscles cannot be explained by a model based only on modulation of Ca^{2+}-sensitivity of the contractile process. To further characterize the specific G-proteins mediating the contractile response of these two different smooth muscle types we used G-protein antibodies developed by Spiegel (Goldsmith et al, 1987, 1988; Simonds et al, 1989; Spiegel et al, 1990; Shenker et al, 1991) and Sternweis (Gutowski et al, 1991; Sternweis et al, 1992). These antisera, raised against synthetic peptides corresponding to the amino acid sequence of the COOH-terminal of G-protein α subunits have been used as effective probes of G-protein structure and function (Spiegel et al, 1990). For example antibodies raised against the COOH-terminal region of the α subunit of Gs and Gi2 have been reported to block receptor-mediated adenylyl cyclase stimulation (Speigel et al, 1990) and inhibition (Simonds et al, 1989), respectively.

As memntioned above, ACh-induced contraction of LES circular muscle results in PLC activation and increased inositol phosphate production. These results are consistent with reports of the involvement of two members of the Gq class of Gα subunits, Gαq and Gα11, in PTX-resistant coupling to phospholipase C activation. Antibodies developed against the COOH-terminal region of the a subunit of Gq-G11 attenuated stimulation of phosphatidyl inositol 4,5-bisphosphate hydrolysis by bradykinin, angiotensin, and histamine in membranes derived from NG108-15 cells, rat liver, and 1321N1 cells, respectively (Gutowski et al, 1991). Futhermore, a novel 42-kD protein, with amino acid sequence identity to Gq, has been purified and found to selectively activate the β1 isozyme of phospholipase C (Pang & Sternweis, 1990; Taylor et al, 1990; Smrcka et al, 1991).

There are some evidences that isozyme of phospholipase C can be regulated by G α subunit and $\beta\gamma$ subunit (Litosch, 1996, 1997). We found that the presence three isozymes of PLC-β1, -β3 and -γ1 in cat LES and esophageal muscles. In other smooth muscles in cat, urinary bladder (Yang et al, 2000), duodenum, and ileum, same three isozymes were detectable (unpublished observations). It was interested that the combination of PLC-β1 and PLC-β3 antibody completely blocked ACh- induced contraction. These two isozymes have synergistically inhibitory effect is, although inhibition degree was a little different, similar to Murthy and Makhlouf's finding that the combined effect of β1 and β3 on PLC activity and intestine muscle contraction by CCK was additive, causing complete inhibition; β1 antibody was more effective (76%) than β3 (24%) antibody (Murthy & Makhlouf, 1995b). They also found

that adenosine A1 receptor mediate PLC-β3 in intestine muscle which can be activated by dual requirement for α and $\beta\gamma$ subunit of G protein (Murthy & Makhlouf, 1995a). Activation of PLC-β1 by calcitonin gene-related peptide involves Gq protein which is insensitive to PTX, but not β subunit. From these findings supports our similar result, since when $G_{q/11}$ antibody was incubated, the inhibitory effect of the incubation of PLC β_3, but not of PLC β_1 was ; in contrast, when G_β antibody was incubated, the inhibitory effect of the incubation of PLC β_1, but not of PLC β_3 was additive. It is strongly possible that $G_{q/11}$ or Gβ may activate different PLC isozyme (PLCβ_1 or PLCβ_3).

However, PLC isozymes was not related to the PKC actiuvation, since DAG-induced contraction was reduced by PLC antibody incubation in LES and esophageal muscle. These data may be interpreted to indicate that PKC dependent contraction, such as ACh induced contraction of Esophagus, or LES spontaneous tone, may be associated with activation of ERKs and p38 Kinase, resulting in activation of HSP 27, possibly through a p38 kinase-mediated pathway. It is unlikely that PKC may directly phosphorylate ERKs (Mennice et al, 1997), more likely PKC may activate Raf, causing it to phosphorylate MEK, which in turn may phosphorylate MAP Kinase (Della Rocca et al, 1997). MAP kinase may then phosphorylate either calponin or caldesmon, or some intermediate protein resulting in caldesmon/calponin phosphorylation. Caldesmon and calponin, when phosphorylated change conformation and no longer inhibit actin-myosin interaction, allowing contraction to occur. It is possible that two distinct parallel pathways may contribute to contraction, one involving p38 kinase and HSP25/27, and the other one involving ERKs and calponin/caldesmon. These sequences of events remain to be resolved, and multiple discrete steps remain to be tested in some detail.

The present study identifies and characterizes the PLC isoforms involved in the contraction from LES and esophageal muscle cells in response to ACh, and its regulation by G-protein a and $\beta\gamma$ subunits. This data suggest that $G_{q/11}$ or Gβ may activate cooperatively different PLC isozyme, PLCβ_1 or PLCβ_3 respectively.

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