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Different Protein Kinase C Isozymes Mediate Lower Esophageal Sphincter Tone and Phasic Contraction of Esophageal Circular Smooth Muscle

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SUMMARY

Circular muscle of the esophagus (ESO) is normally relaxed and contracts phasically in response to neural stimuli. In contrast, lower esophageal sphincter (LES) circular muscle maintains spontaneous tone and relaxes in response to neural stimuli. We have previously shown that *in vitro,* spontaneous LES tone and contraction of ESO in response to acetylcholine (ACh) are antagonized by protein kinase C (PKC) inhibitors, suggesting that PKC activation is responsible for these functions. In the current study, Western blot analysis of LES and ESO revealed PKC-α, -βII, and -γ isozymes in LES circular muscle, but only PKC-βII translocated from the cytosolic to the membrane fraction in response to ACh. In contrast, ESO contained PKC-βII, -γ, and -ϵ, and only PKC-ϵ translocated to the membrane fraction in response to ACh. In LES single cells isolated by enzymatic digestion and permeabilized by saponin, 1–2-dioctanoylglycerol-mediated contraction was inhibited by preincubation with PKC-βII antiserum but not by other PKC antisera. In esophageal cells, contraction was inhibited by the PKC-ϵ antiserum but not by antisera against other PKC isozymes. *N*-Myristoylated peptides derived from the pseudosubstrate sequences of PKC isozymes were used to inhibit saponin, 1–2-dioctanoylglycerol-induced contraction of LES and ESO smooth muscle cells. Contraction of LES cells was reduced by the $\alpha\beta\gamma$ pseudosubstrate but not by the α, δ, or ϵ pseudosubstrate. Contraction of ESO cells was reduced by the ϵ pseudosubstrate but not by the α, δ, or $\alpha\beta\gamma$ pseudosubstrate. We conclude that different types of contractile activity in the ESO and LES are mediated by different PKC isozymes. LES contraction is mediated by the calcium-dependent PKC-βII, whereas contraction of ESO is mediated by the calcium-independent PKC-ϵ.

ESO circular smooth muscle is normally relaxed and contracts with a brief and powerful "phasic" contraction in response to neural (cholinergic) stimuli induced by swallowing. LES circular muscle maintains spontaneous myogenic tone and relaxes in response to neural (nonadrenergic-noncholinergic inhibitory) stimuli.

We have previously shown that both ESO contraction in response to a maximally effective dose of ACh and LES tone are mediated by PKC-dependent intracellular transduction pathways. ACh-induced contraction of ESO circular smooth muscle requires the influx of extracellular calcium and activation of PKC and is independent of calmodulin (1, 2). In addition, the calcium influx is required primarily for the activation of the phospholipases responsible for the production of DAG and not for the direct activation of PKC (2). In contrast, LES tone is mediated by a calcium-dependent PKC pathway. During the maintenance of tone, spontaneous phospholipase C activity produces low levels of Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3 causes the release of low levels of calcium from intracellular stores (3). However, different PKC isozymes may regulate these two contractile processes in these distinct tissue types.

PKC is a family of homologous serine and threonine protein kinases. Eleven isozymes of PKC have been identified in mammalian tissues. These isozymes can be divided into three groups based on their calcium and phospholipid requirements for activation: classic, or conventional PKC

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ABBREVIATIONS: ESO, esophagus; ACh, acetylcholine; ANOVA, analysis of variance; DAG, dioctanoylglycerol; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; LES, lower esophageal sphincter; NC, nitrocellulose; PKC, protein kinase C; PKI, protein kinase I; RACK, receptor for activated C kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(α-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKA, protein kinase A.

(cPKC), including α, βI, βII, and γ, which are calcium and phospholipid dependent; new PKC (nPKC), including δ, ϵ, η, θ, and μ, which are calcium independent and phospholipid dependent; and atypical PKC (aPKC), including ζ and λ, which are calcium and phospholipid independent (4).

In the current study, we examined the PKC isozymes present in the ESO and LES and investigated which ones mediate LES basal tone and ACh-induced contraction of ESO. We found that LES contraction is mediated by the calcium-dependent PKC-β isozymes, whereas contraction of the ESO is mediated by the calcium-independent PKC-ϵ.

Materials and Methods

Tissue dissection and dispersion of smooth muscle cells. Adult cats of either sex weighing 3–5 kg were used, and ESO and LES smooth muscle squares were prepared as previously described (1, 5). The chest and abdomen were opened with a midline incision exposing the ESO and stomach. The ESO and stomach were removed together and pinned onto a wax block at their *in vivo* dimensions and orientation. The ESO and stomach were opened along the lesser curvature. The location of the squamocolumnar junction was identified, and the mucosa was peeled. The high pressure zone is characterized by a visible thickening of the circular muscle layer in correspondence of the squamocolumnar junction and immediately proximal to the sling fibers of the stomach. We have previously shown that a 5–8-mm band of tissue that coincides with the thickened area constitutes the LES and has distinct characteristics when examined *in vivo*, in the organ bath, or as single cells after enzymatic digestion (1, 6).

After the ESO and stomach were opened and the LES was identified as described above, 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 ESO was discarded to avoid overlap. The circular muscle layer from LES and ESO 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; then, the slices were cut by hand into 2 × 2-mm tissue squares. Tissue squares of circular muscle were used either in Western blot analysis or further digested to isolated single cells for contractility studies.

Tissue squares were digested in HEPES buffer containing 0.1% collagenase type II to isolate smooth muscle cells as previously described (1); the HEPES solution contained 115 mM NaCl, 5.8 mM KCl, 12 mM KH_2PO_4, 2.5 mM glucose, 25 mM HEPES, 2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.3 mg/ml BME amino acid supplement, and 0.09 mg/ml soybean trypsin inhibitor. The solution was gently gassed with 100% O_2. At the end of the digestion period, the tissue was poured over a 450-μm nylon mesh (Tetko, Elmsford, NY), rinsed in collagenase-free HEPES buffer to remove any trace of collagenase, and then incubated in this solution at 31° and gassed with 100% O_2. The cells were allowed to dissociate freely for 10–20 min.

Cells were permeabilized, when required, to control intracellular calcium concentration and to allow the use of agents such as PKC antibodies, which do not diffuse across the intact cell membrane. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with an enzyme-free cytosolic buffer composed of 20 mM NaCl, 100 mM KCl, 5.0 mM $MgSO_4$, 0.96 mM NaH_2PO_4, 1.0 mM EGTA, 0.48 mM $CaCl_2$, and 2% bovine serum albumin. The cytosolic buffer was equilibrated with 95% O_2/5% CO_2 to maintain pH 7.2 at 31°. Muscle cells dispersed spontaneously in this medium. After dispersion, the cells were permeabilized by incubation for 3 min in cytosolic buffer containing 75 μg/ml saponin. After exposure to saponin, the cell suspension was spun at 500 × *g*, and the resulting pellet was resuspended in saponin-free modified cytosolic buffer containing 10 μM antimycin A, 1.5 mM ATP, and an ATP-regenerating system consisting of 5 mM creatine phosphate and 10 units/ml creatine phosphokinase. After the cells were washed free of saponin, they were resuspended in modified cytosolic buffer.

Agonist-induced contraction of isolated muscle cells. The cells were contracted by exposure for 30 sec to DAG (10^{-7} M for ESO and 10^{-6} M for LES) in permeabilized cells. These concentrations produce a maximal contraction of the circular smooth muscle from these two distinct tissue types.

When PKC antibodies were used, permeabilized cells were incubated with the antibody at a 1:200 dilution for 60 min before the addition of DAG (7, 8). To demonstrate the selectivity of the antibodies, permeabilized cells were also incubated with antibody in combination with the peptide against which the antibody was prepared. Peptide and antibody (1:200 dilution for each) were added 60 min before the addition of DAG.

The inhibitors used in this study were *N*-myristoylated peptides derived from the pseudosubstrate sequences of PKC isozymes (Table 1). *N*-Myristoylated peptides can diffuse across the cell membrane; therefore, intact cells were incubated with 10^{-7} M peptide for 60 min before the addition of DAG. We have previously shown that preincubation of lacrimal gland acini for 60 min with 10^{-7} M myr-PKC-α results in 80% inhibition of protein secretion by phorbol esters (9). A myristoylated peptide derived from the sequence of the endogenous inhibitor of cAMP-dependent protein kinase A, indicated in the figure as PKI, was used as a negative control to test the sequence specificity of the PKC pseudosubstrate peptides.

After exposure to DAG, 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 and covered by a coverslip, and the edges were sealed with nail enamel to prevent evaporation.

The length of 30 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, Cupertino, CA) with an image analysis software program (Image 1.33; National Institutes of Health, Bethesda, MD). Contraction was expressed as percent shortening of average of 30 consecutive cells compared with control. The average control cell length was 72 ± 1 μm in LES and 73 ± 1 μm (42 measurements) in ESO.

Polyacrylamide gel electrophoresis and immunoblotting. The identification of PKC isozymes in LES and ESO circular muscle was performed by Western blot analysis. Briefly, tissue squares of LES and ESO circular muscle were homogenized in phosphate buffer and centrifuged for 1 min at low rpm. The supernatant was mixed with SDS buffer, boiled for 5 min, and centrifuged at 12,000 rpm for 5 min at 4°. Prestained molecular mass marker was prepared in the same manner. After these supernatant samples were subjected to SDS-polyacrylamide gel electrophoresis (10), the separated proteins

TABLE 1
Structure of the synthetic *N*-myristoylated pseudosubstrate peptides

myr-PKC$\alpha\beta\gamma$ (20–28): Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln
myr-PKCα (15–28): Myr-Asp-Val-Ala-Asn-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln
myr-PKCϵ (149–164): Myr-Glu-Arg-Met-Arg-Pro-Arg-Lys-Arg-Gln-Gly-Ala-Val-Arg-Arg-Arg-Val
myr-PKCδ (142–153): Myr-Met-Asn-Arg-Arg-Gly-Ala-Ile-Lys-Gln-Ala-Lys-Ile
myr-PKI (17–25): Myr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile

were electrophoretically transferred to an NC membrane (BioRad, Melville, NY) at 60 V for 5 hr, followed by 30 V overnight. Transfer of proteins to the NC membrane was confirmed with Ponseau S staining reagent. To block nonspecific binding, the NC membrane was incubated in 5% nonfat dry milk in phosphate-buffered saline for 60 min, followed by three rinses in milk-free buffer. Incubation with 1:1000 dilution of antibody raised against each PKC isozyme (i.e., α, βI, βII, γ, δ, ϵ, ζ) was done for 1 hr with shaking, followed by three washes with antibody-free buffer. This was followed by a 60-min incubation in horseradish peroxidase-conjugated goat anti-rabbit antibody. Detection was achieved with an enhanced chemiluminescence agent. Molecular mass was estimated by comparison of sample bands with a prestained molecular mass marker.

We examined the ACh-stimulated translocation of PKC isozymes from the cytosol to the membrane fraction of ESO and LES smooth muscle. Tissue squares (150 mg) were equilibrated in 400 μl of Krebs' solution and gassed with 95% O_2/5% CO_2, at 37° for 20 min. For measurement of agonist-stimulated translocation, tissue squares were exposed to ACh (10^{-7} M for LES and 10^{-5} M for ESO). These concentrations of ACh have previously been determined to produce maximal PKC-dependent contraction in the two different tissue types. After 30 sec, the reaction was stopped with 10 volumes of ice-cold Krebs' solution. Tissue squares were collected and homogenized in 20 mM Tris buffer, pH 7.4, containing 0.5 M EDTA, 0.5 M EGTA, 10 mg/ml leupeptin, 10 mg/ml aprotinin, and 10 mM β-mercaptoethanol. Homogenization consisted of two 10-sec bursts with a Tissue Tearer (Biospec, Racine, WI) followed by 40–60 strokes with a Dounce tissue grinder (Wheaton, Melville, NJ). Samples were centrifuged at 100,000 $\times$ *g* for 40 min at 4° (50 Ti rotor; Beckman Ultracentrifuge, Palo Alto, CA). The supernatant was collected and, after the addition of 30 μl of 10 mg/ml phenylmethylsulfonyl fluoride and a 30-min incubation, was used as the cytosolic fraction. The pellet was resuspended in 3 ml of homogenizing buffer containing 0.1% Triton X-100 and rehomogenized by 20 strokes with a Dounce tissue grinder. The resuspended sample was mixed well by the use of a tube rotator for 30–40 min at 4° and then centrifuged at 100,000 $\times$ *g* for 50 min at 4°. The supernatant of this centrifugation was collected as the membrane fraction. The identification of PKC isozymes in the cytosolic and membrane fractions was performed by Western blot analysis as described above. The bands were analyzed using scanning densitometry (Howtek, Hudson, NH).

In addition to Western blot analysis, ACh-stimulated translocation of PKC activity was measured by colorimetric assay. The cytosolic and membrane fractions were immunoprecipitated by isozyme-specific PKC antibodies, and PKC activity was measured. Briefly, 5 μg of isozyme-specific PKC antibodies (PKC-βII for LES and PKC-ϵ for ESO) were added to 400 μl of cytosolic or membrane fraction and incubated for 1 hr at 4°. Then, 40 μl of protein A/G PLUS-agarose (Santa Cruz Biochemicals, Santa Cruz, CA) was added, and samples were incubated at 4° with rocking. After 1 hr, samples were microcentrifuged (Fisher Scientific, Pittsburgh, PA) for 15–20 sec at 4°. The pellet was suspended in 20 μl of RIFA buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1% tergitol, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) and solubilized. The supernatant was removed, and the pellet was suspended in 20 μl of RIFA buffer and solubilized. Ten microliters of solubilized sample was used in the PKC colorimetric assay.

Measurement of PKC activity. PKC activity of immunoprecipitated smooth muscle of the LES and ESO was measured using the Pierce Colorimetric PKC Assay no. 29510 (Pierce, Rockford, IL). Briefly, a peptide substrate that was labeled with 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 quantified by measurement of its absorbance at 570 nm.

Protein determination. Protein content was obtained after hydrolysis by 0.1 N NaOH at 80° to solubilize the protein, followed by neutralization with HC1. The amount of protein present was determined by colorimetric analysis (BioRad Protein Assay; BioRad, Richmond, CA) according to the method of Bradford (11).

Pseudosubstrate peptide synthesis. Myristoylated peptides were synthesized by BOC strategy on an MHBA resin (Novabiochem, Meudon, France) using an Applied Biosystems 430A automated synthesizer (Foster City, CA). Protocols and reagents were used as recommended by the manufacturer. Myristic acid was coupled to the peptide using dicyclohexylcarbdiimide hydroxybenzotriazole. Peptides were purified by reverse-phase high performance liquid chromatography on a Vydac C_4 (30 $\times$ 0.9-cm) preparative column using a trifluoroacetic acid/acetonitrile solvent system. Peptide integrity was monitored by amino acid analysis and mass spectrometry.

Drugs and chemicals. PKC antibodies (i.e., α, βI, βII, γ, δ, ϵ, ζ) were from GIBCO BRL (Gaithersburg, MD). Enhanced chemiluminescence agents and rainbow-prestained molecular mass markers were from Amersham (Arlington Heights, IL). Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Pierce. Collagenase type II and soybean trypsin inhibitor were from Worthington Biochemicals (Freehold, NJ). SDS sample buffer was from BioRad (Hercules, CA). Saponin, Ponseau S, BME amino acid supplement, EGTA, HEPES, creatine phosphate, creatine phosphokinase, ATP, antimycin A, and other reagents were purchased from Sigma Chemical (St. Louis, MO).

Statistical analysis. Estimates of the half-maximal response to the myristoylated pseudosubstrate peptides were determined by interpolation from graphs of log concentration versus logit values of percent shortening (12). Data are expressed as mean $\pm$ standard error. Statistical differences between groups were determined by Student's *t* test. Differences between multiple groups were tested using ANOVA for repeated measures and checked for significance using Scheffé's *F* test.

Results

Western blot analysis of PKC isozymes. Fig. 1 shows that PKC isozymes, detected by Western blot analysis in the circular layer of LES smooth muscle, include the calcium-dependent PKC-α, -βII, and -γ isozymes. In contrast, the PKC-βII, -γ, and -ϵ isozymes are present in the circular smooth muscle of the ESO. The specificity of each immunoreactive band was substantiated by the blockade of the signal after incubation of the corresponding peptide antigen together with the antibody directed against each PKC isozyme (Fig. 1, *lane 1*).

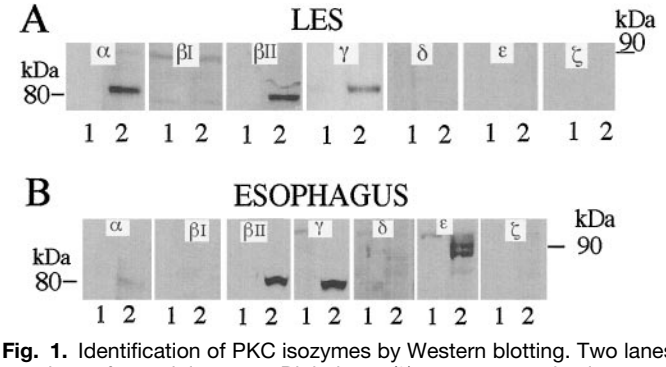

Fig. 1. Identification of PKC isozymes by Western blotting. Two lanes are shown for each isozyme. *Right lanes (2),* were exposed to isozyme-specific PKC antibodies. A, PKC- α, -βII, and -γ immunoreactive bands were detected in LES circular smooth muscle. B, PKC-βII, -γ, and -ϵ immunoreactive bands were detected in ESO circular smooth muscle. *Left lanes (1),* were exposed to PKC-specific antibodies in the presence of their isozyme-specific peptide. The absence of any band in *lane 1* supports the specificity of each immunoreactive band.

Different PKC isozymes translocate in response to ACh in LES and ESO. Activation of PKC is associated with the translocation of the enzyme from the cytosol to the membrane fraction (13, 14). To test the functional significance of the PKC isozymes present in LES and ESO circular smooth muscle, we examined their translocation from cytosol to membrane in response to ACh stimulation.

Western blot analysis revealed that among the PKC isozymes present in LES circular smooth muscle, only PKC-βII translocates from the cytosol (Fig. 2, *lane 1*) to the membrane (Fig. 2, *lane 4*) in response to ACh stimulation. In ESO circular smooth muscle, only PKC-ϵ translocates from the cytosol to the membrane in response to ACh.

The bands of membrane and cytosolic fraction were analyzed using scanning densitometry (Fig. 3A). In unstimulated control LES, 72% of the total density of the PKC-βII bands was present in the cytosolic fraction, whereas the remaining 28% was found in the membrane. After ACh stimulation, these proportions reverse, with 70% of total PKC-βII band density present in the membrane fraction and 30% density remaining in the cytosol. In the ESO under control conditions, 72% of the total density of the PKC-ϵ bands was present in the cytosolic fraction and 28% was in the membrane fraction. After ACh stimulation, 68% of total PKC-ϵ band density was present in the membrane fraction, with 30% density remaining in the cytosol. Fig. 3B demonstrates that the ratio of density (membrane/cytosol) of PKC-βII in the LES and PKC-ϵ in the ESO significantly increases after ACh stimulation ($p < 0.01$, paired t test).

In addition to Western blot analysis, ACh-stimulated PKC translocation was examined by measurement of PKC activity (Fig. 3C). The cytosolic and membrane fractions were immunoprecipitated with isoenzyme-specific antibodies (βII for LES and ϵ for ESO), and PKC activity was measured by colorimetric assay. The ratios of PKC activity (membrane/cytosol) of the βII isozyme for the LES and the ϵ isozyme for the ESO were significantly increased after ACh stimulation ($p < 0.01$, paired t test).

Inhibition of DAG-induced contraction of permeabilized LES and ESO cells by PKC antibodies. To confirm that PKC-mediated contraction may be isozyme specific, we examined the effect of different PKC antibodies on contraction induced by the endogenous PKC activator DAG .

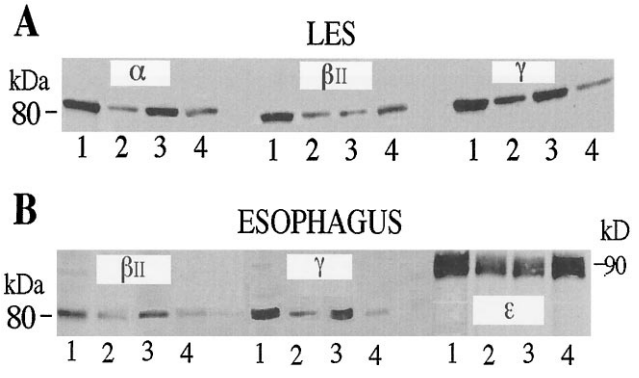

Fig. 2. Western blot identification of PKC isozymes translocating from the cytosol to the membrane of LES and ESO circular smooth muscle in response to ACh. *Lanes 1 and 2*, cytosolic and membrane fractions of unstimulated samples. *Lanes 3 and 4*, cytosolic and membrane fractions of ACh-simulated samples. A, Among the three isozymes present in the LES circular smooth muscle (α, βII, and γ), only PKC-βII translocated from the cytosol to the membrane in response to ACh. B, Among the three isozymes present in the ESO circular smooth muscle (βII, γ, and ϵ), only PKC-ϵ translocated from the cytosol to the membrane in response to ACh.

Fig. 4A shows that DAG-induced contraction of permeabilized LES cells was significantly inhibited by antibodies raised against PKC-βII and not by antibodies raised against the PKC-γ, -ϵ, or -α isozyme (ANOVA, $p < 0.01$). Inhibition of DAG-induced contraction PKC-βII was concentration dependent (Fig. 4B) and reversed by the addition of the PKC-βII-specific peptide. Fig. 5A shows that contraction of permeabilized ESO cells was inhibited by antibodies raised against PKC-ϵ and not by antibodies raised against the PKC-βII, -γ, or -α isozyme (ANOVA, $p < 0.01$). Inhibition of DAG-induced contraction PKC-ϵ was concentration dependent and reversed by the addition of the PKC-ϵ-specific peptide (Fig. 5B). These data support the translocation experiments and suggest that PKC-βII may mediate contraction of LES, whereas PKC-ϵ may mediate contraction of ESO.

Different *N*-myristoylated pseudosubstrate peptides inhibit DAG-induced contraction of LES and ESO. To characterize further the specific PKC isozymes that mediate contraction of these two different smooth muscle types, we used *N*-myristoylated peptides derived from the pseudosubstrate sequences of PKC-αβγ, -α, -δ, and -ϵ (myr-PKC-αβγ, myr-PKC-α, myr-PKC-δ, and myr-PKϵ) (Table 1) and examined their effect on DAG-induced contraction of intact LES and ESO smooth muscle cells.

DAG-induced contraction of LES cells was inhibited by the myristoylated peptide corresponding to the pseudosubstrate sequence of PKC-αβγ (myr-PKC-αβγ) and was not inhibited by myr-PKC-α, myr-PKC-δ, or myr-PKC-ϵ (Fig. 6A). In contrast, DAG-induced contraction of ESO was inhibited by myr-PKC-ϵ and was not inhibited by myr-PKC-α, myr-PKC-αβγ, or myr-PKC-δ. PKI had no effect on either tissue type (Fig. 6B). These data support the hypothesis that PKC-β mediates contraction of LES, whereas PKC-ϵ mediates contraction of ESO.

The dose response of the *N*-myristoylated pseudosubstrate peptides on DAG-induced contraction was examined (Fig. 7). DAG-induced contraction was dose-dependently inhibited by myr-PKC-αβγ in the LES and by myr-PKC-ϵ in the ESO. The half-maximal response, calculated by logit transformation, was seen at 3×10^{-10} M myr-PKC-ϵ for ESO and 4×10^{-10} M myr-PKC-αβγ for LES.

Discussion

PKC plays an important role in the signal transduction pathway mediating smooth muscle contraction (15–21). We have previously described PKC-dependent contraction in the circular smooth muscle of both the LES and ESO. In the LES, we found that *in vivo* tone is reduced by the PKC antagonists H7 and calphostin C but not by the calmodulin antagonist W7. We have demonstrated that LES tone is associated with the spontaneous activity of phosphatidylinositol-specific phospholipase C, resulting in submaximal production of Ins(1,4,5)P_3 and DAG, which act synergistically to activate PKC in a calcium-dependent manner (3). Furthermore, we have shown that this PKC-mediated contraction is augmented by additional DAG production arising from hydrolysis of phosphatidylcholine by phosphatidylcholine-specific phospholipases C and D (19).

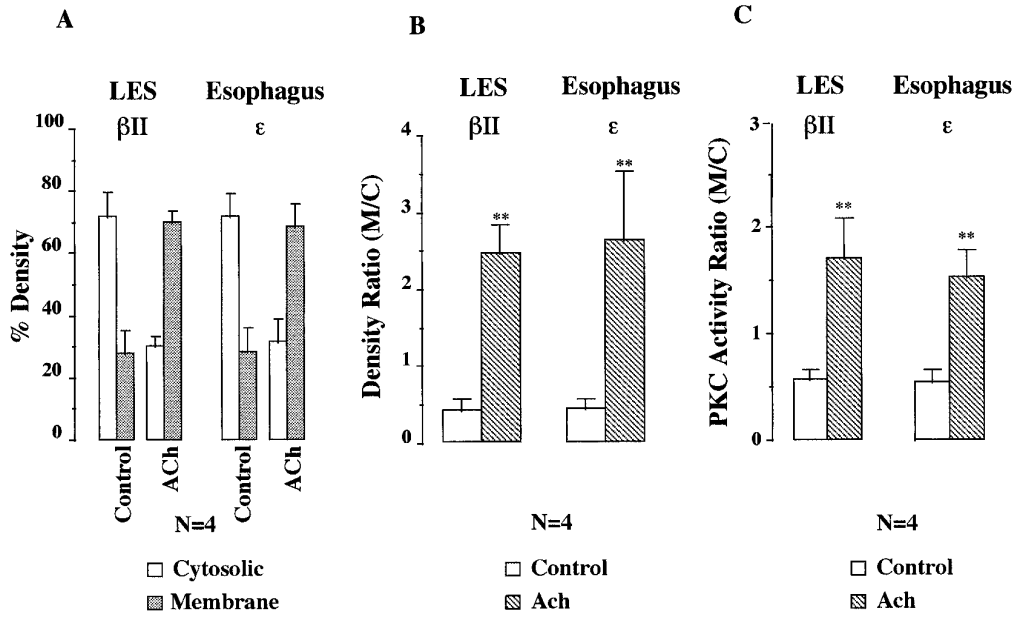

Fig. 3. ACh stimulates the translocation of PKC-βII in LES and PKC-ϵ in ESO smooth muscle. A, Western blot analysis was performed, and bands of membrane and cytosolic fraction were analyzed using scanning densitometry. In unstimulated LES (*Control*), 72% of the total density of the PKC-βII bands was present in the cytosolic fraction, whereas the remaining 28% was found in the membrane. After ACh stimulation, these proportions reverse, with 70% of total PKC-βII band density present in the membrane fraction, and 30% of the density remaining in the cytosol. In the unstimulated ESO (*Control*), 72% of the total density of the PKC-ϵ bands was present in the cytosolic fraction, and 28% was in the membrane fraction. After ACh stimulation, 68% of total PKC-ϵ band density was present in the membrane fraction, with 30% density remaining in the cytosol. Values are mean ± standard error from four animals. B, The ratio of density (membrane/cytosol) before and after ACh stimulation is shown for PKC-βII in the LES and PKC-ϵ in the ESO. Density ratios were derived from the data shown in A. Membrane/cytosol density ratios increased significantly after ACh stimulation ($p < 0.01$, paired *t* test). Values are mean ± standard error from four animals. C, The ratio of activity (membrane/cytosol) of the βII isozyme for the LES and the ϵ isozyme for the ESO was significantly increased after ACh stimulation ($p < 0.01$, paired *t* test). PKC activity was measured by colorimetric assay using cytosolic and membrane fractions immunoprecipitated with isozyme-specific antibodies (βII for LES and ϵ for ESO). Values are mean ± standard error from four animals.

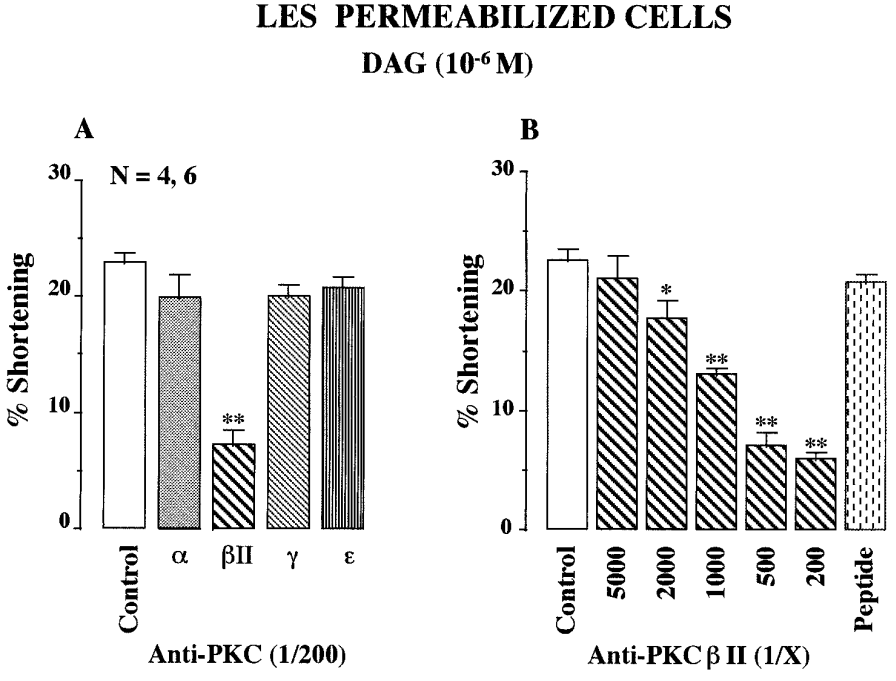

Fig. 4. PKC-βII mediates DAG-induced contraction of permeabilized circular muscle cells of LES. A, DAG-induced contraction of LES cells was significantly inhibited by antibodies raised against PKC-βII and not by antibodies raised against the PKC-γ, -ϵ, or -α isozymes ($p < 0.01$, ANOVA). B, DAG-induced contraction was dose-dependently inhibited by PKC-βII antibody, with maximal inhibition at a dilution of 1:200. The inhibition was reversed by the addition of the PKC-βII-specific peptide. Values are mean ± standard error from four to six animals with 30 cells counted at random for each *data point*.

In the ESO, we have shown that ACh-induced contraction requires the influx of extracellular calcium and activation of PKC and is independent of calmodulin because it is inhibited by PKC inhibitors such as H7, calphostin C, and chelerythrine and not affected by calmodulin inhibitors (1, 2). ESO contraction requires activation of phosphatidylcholine-specific phospholipase C (20), phospholipase D, and phospholipase A_2 (21) and production of DAG and arachidonic acid without an associated increase in Ins(1,4,5)P_3 (2, 8). In addition, we have shown that DAG-induced contraction of ESO is

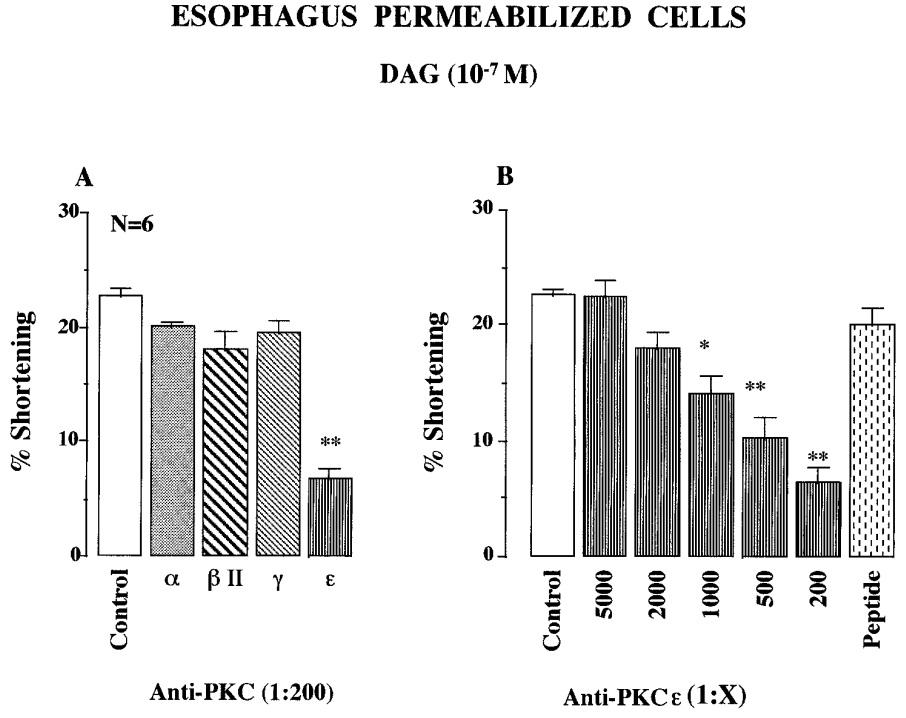

Fig. 5. PKC-ϵ mediates DAG-induced contraction of permeabilized circular muscle cells the ESO. A, DAG-induced contraction of ESO cells was significantly inhibited by antibodies raised against PKC-ϵ and not by antibodies raised against the PKC-βII, -γ, or -α isozymes ($p < 0.01$, ANOVA). B, DAG-induced contraction was dose-dependently inhibited by PKC-ϵ antibody, with maximal inhibition at a dilution of 1:200. The inhibition was reversed by the addition of the PKC-ϵ-specific peptide. Values are mean ± standard error from six animals with 30 cells counted at random for each *data point*.

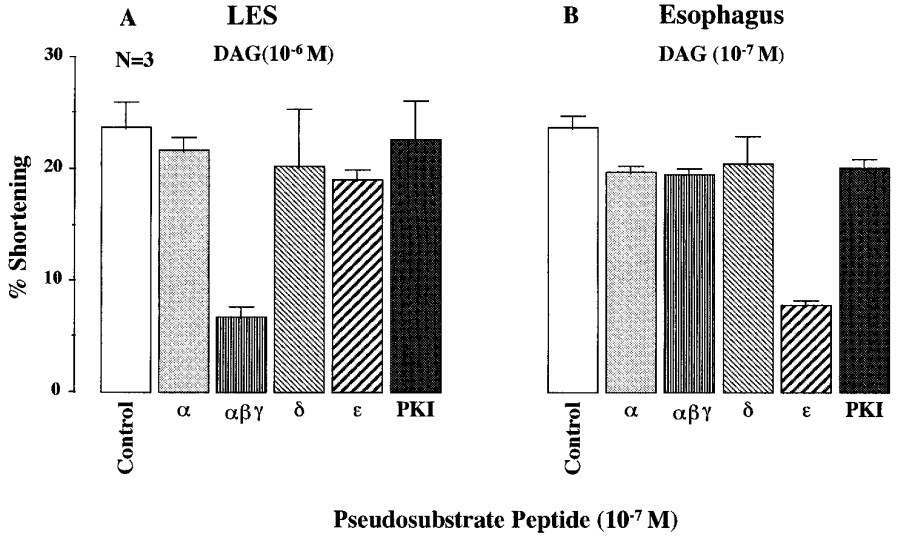

Fig. 6. The effect of myristoylated pseudosubstrate peptides on DAG-induced contraction of intact smooth muscle cells from the circular layer of the LES and ESO. A, DAG-induced contraction of LES cells was significantly inhibited by the myristoylated peptide corresponding to the pseudosubstrate sequence of PKC-$\alpha\beta\gamma$ (myr-PKC-$\alpha\beta\gamma$) and not inhibited by myr-PKC-α, myr-PKC-δ, or myr-PKC-ϵ ($p < 0.01$, ANOVA). B, In contrast, DAG-induced contraction of ESO was significantly inhibited by myr-PKC-ϵ and not inhibited by myr-PKC-α, myr-PKC-$\alpha\beta\gamma$, or myr-PKC-δ ($p < 0.01$, ANOVA). The PKA inhibitor, PKI, had no effect on either tissue type. These data suggest that PKC-β may mediate contraction of LES, whereas PKC-ϵ may mediate contraction of ESO. Values are mean ± standard error of three animals with 30 cells counted at random for each *data point*.

independent of cytosolic calcium, suggesting that the calcium influx is required primarily for the activation of the phospholipases responsible for the production of DAG and not for activation of PKC (2).

The results of the current study suggest that although both contractile processes are PKC-dependent, different PKC isozymes (one calcium-dependent and one calcium-independent) may mediate contraction in these two distinct tissue types. Calcium-dependent PKC isozymes (α, βI, βII, and γ) have a calcium-binding domain that allows calcium and DAG to act synergistically on these PKC isozymes, whereas calcium-independent isozymes (δ, ϵ, ζ, η, and θ) lack this calcium-binding domain and thus do not require calcium for activation (22–24). In the current study, we showed that contraction of the circular smooth muscle of the LES is mediated by the calcium-dependent PKC-βII isozyme, whereas contraction of the circular smooth muscle of the ESO is mediated by the calcium-independent PKC-ϵ isozyme. These conclusions arise from the following findings.

Expression of PKC isozymes in LES and ESO smooth muscle. Using isoenzyme-specific antibodies in Western blotting experiments, we demonstrate the presence of PKC-α, -βII, and -γ isozymes in the circular smooth muscle layer of LES and of PKC-βII, -γ, and -ϵ isozymes in the ESO. These results are consistent with numerous studies that report the expression of multiple PKC isozymes in smooth muscle. Multiple PKC isozymes have been reported in rat aorta (25, 26), ferret aorta (27), human saphenous vein and renal artery (28), hamster vas deferens (28), and rat mesenteric artery (29). The functional significance of the individual PKC

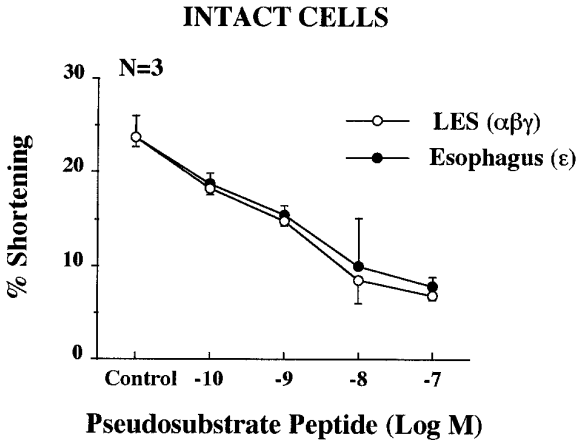

Fig. 7. DAG-induced contraction was dose-dependently inhibited by myr-PKC-$\alpha\beta\gamma$ in the LES and by myr-PKC-ϵ in the ESO. Values are mean ± standard error of three animals with 30 cells counted at random for each *data point*.

isozymes expressed in the LES and ESO was examined in our translocation and contractility experiments.

ACh stimulation causes PKC-βII translocation in LES and PKC-ϵ translocation in ESO. In the current study, we demonstrated by two distinct techniques that in LES, the calcium-dependent PKC-βII translocates from the cytosol to the membrane in response to ACh, whereas in ESO, the calcium-independent PKC-ϵ translocates in response to ACh.

Calcium-dependent and -independent PKC isozymes have been shown to translocate from the cytosol to the membrane on appropriate stimulation of a variety of cell types, including smooth muscle (14, 15). The subcellular distribution of PKC in single smooth muscle cells from the ferret portal vein has been imaged using a fluorescent (non-isozyme-specific) PKC probe showing that a calcium-dependent PKC isozyme relocates from the cytosol to the surface membrane during PKC-mediated contraction (14). In addition, the calcium-independent PKC-ϵ and PKC-ζ isozymes were identified in ferret aorta and shown to translocate on agonist stimulation (27). It has been proposed that translocation of PKC reflects PKC binding to intracellular receptors in the particulate fraction (RACKs) and that binding to RACKs may be required for PKC-mediated function. PKC binding to RACKs is specific, dose dependent, and saturable and may confer specificity of isozyme action by differential localization of isozyme-specific RACKs (30, 31).

We used Western blotting to examine the relative proportion of isozymes in the cytosol and membrane before and after ACh stimulation. It is notable that despite the presence of several distinct PKC isozymes in LES, the membrane-to-cytosol density ratio changed only for PKC-βII (from 0.42 ± 0.15 to 2.44 ± 0.39). Similarly, in ESO, the ratio changed only for PKC-ϵ (from 0.43 ± 0.14 to 2.61 ± 0.9).

These data are confirmed by activity measurements, which show that the change in activity ratios of PKC-βII in the LES and of PKC-ϵ in the ESO is similar to the change in Western blot measurements of density ratio.

We conclude that the translocating PKC-βII in the LES and PKC-ϵ in the ESO are the isozymes that may mediate contraction in these two tissue types, possibly by bringing the enzymes to their specific RACKs or membrane-bound effector proteins. The function of the other isozymes is not presently known; they may participate in other cellular functions.

DAG-induced contraction of LES and ESO cells is inhibited by isozyme-specific PKC antibodies and isozyme-specific pseudosubstrate-derived peptides. We found that DAG-induced contraction of permeabilized smooth muscle cells was dose-dependently inhibited by antibodies raised against PKC-βII in the LES and PKC-ϵ in the ESO. In the LES, a complete concentration-response curve was generated only for PKC-βII because it was the only isozyme tested that significantly inhibited contraction in the LES. Similarly, in ESO, a complete concentration-response curve was generated only for PKC-ϵ because it was the only isozyme tested that significantly inhibited contraction in the ESO. In general, in this and other experiments using antibodies, we found a maximal effect at a 1:200 concentration.

The mechanism through which these antibodies inhibit contraction is unclear. PKC is composed of four conserved (C_1–C_4) and five variable regions (V_1–V_5), generally organized in the sequence V1-C1-V2-C2-V3-C3-V4-C4-V5. C_1 and C_2 are contained in the regulatory domain and contain the binding sites for phosphatidylserine, calcium, DAG, phorbol esters, and calphostin C. C_3 and C_4 are contained in the catalytic domain and contain the binding sites for ATP and for the different PKC substrates (23). V_3 contains a protease-sensitive hinge region that allows the molecule to fold in such a way as to allow the C_1 region of the regulatory domain to interact with the C_4 region of the catalytic domain, keeping the enzyme inactive in resting cells. The new PKC isozymes, including δ ϵ, η, θ, and μ, lack the C_2 region, which has been implicated in the regulation of PKC by calcium (32–34).

Antibodies were raised against synthetic peptides containing sequences unique to specific PKCs. PKC-α and PKC-γ antibodies were derived from peptide sequences from the variable V_3 region, and PKC-βII and PKC-ϵ antibodies were derived from peptide sequences from the V_5 region. The V_5 region of the PKC molecule is located at the carboxyl terminus on the catalytic domain, adjacent to the substrate binding site. It is possible that the binding of anti-PKC-βII and anti-PKC-ϵ to peptide sequences in the V_5 region induces a conformational change in the molecule that inhibits substrate binding. Alternatively, antibody binding may inhibit PKC activity by inhibiting the phosphorylation of the kinase itself. There exists some evidence of phosphorylation of PKC in the V_1, V_2, and V_5 regions, which is required for PKC catalytic activity (35–37). Most likely, however, the precise site of binding of the antibody to a specific PKC is not absolutely important because immunoglobulins are much larger molecules (180–500 kDa) than PKC (80–90 kDa) and may affect the structural conformation of the enzyme, regardless of their specific binding site.

These data support the translocation studies and suggest that PKC-βII mediates contraction in the LES and that PKC-ϵ mediates contraction in the ESO. This view is further supported by the finding that isozyme-specific pseudosubstrate-derived peptides inhibit DAG-induced contraction in the LES and ESO. All PKC isozymes contain an autoinhibitory sequence, near the C_1 domain, called the pseudosubstrate domain that is thought to interact with the catalytic domain to keep the enzyme inactive in resting cells. Allosteric activators, such as DAG or phorbol esters, relieve this intramolecular control by inducing a conformational change

in the molecule that liberates the substrate binding domain from the pseudosubstrate, thereby activating the enzyme (38). Synthetic peptides based on the pseudosubstrate sequences of individual isozymes might be specific inhibitors because they exploit the substrate specificity of the enzyme without interfering with ATP binding. A recent approach uses modification of peptides by myristoylation to overcome the permeability barrier of the plasma membrane. Myristoylated peptides that are based on the pseudosubstrate sequence of PKC-α and PKC-β have been reported to inhibit the PKC-mediated phosphorylation of the myristoylated alanine-rich C kinase substrate protein (MARCKS) and phospholipase D activation of human fibroblasts (39). We have previously shown that preincubation of lacrimal gland acini for 60 min with 10^{-7} M myr-PKC-α results in 80% inhibition of protein secretion by phorbol ester (9). In a recent study, we reported that synthetic myristoylated peptides derived from the pseudosubstrate sequences of PKC-α, -δ, and -ϵ, three isoforms that are present in lacrimal gland acini, inhibit phorbol ester- and cholinergic agonist-induced protein secretion in a concentration-dependent manner. We showed that these peptides neither interfered with cholinergic-induced changes in cytosolic Ca^{2+} concentration nor inhibited vasoactive intestinal peptide-induced protein secretion, a response mediated by cAMP and protein kinase A. Although these peptides did not show clear selectivity when tested *in vitro* with purified recombinant PKCs, we showed that they differentially affected phorbol ester- and agonist-induced protein secretion in the lacrimal gland. We suggested that *in vivo*, these peptides might be inhibiting (competing for) the binding of their respective PKC isoform to crucial intracellular receptors such as RACKs rather than inhibiting PKC activity.

In the current study, we demonstrated isozyme-specific inhibition of DAG-induced contraction of intact smooth muscle cells from the LES and ESO. DAG-induced contraction of LES cells was significantly inhibited by the myristoylated peptide corresponding to the pseudosubstrate sequence of PKC-$\alpha\beta\gamma$ (myr-PKC-$\alpha\beta\gamma$); whereas ESO contraction was significantly inhibited by myr-PKC-ϵ. It is worth noting that myr-PKC-$\alpha\beta\gamma$ differs from myr-PKC-α by the addition to its amino terminus of only five amino acids (Table 1). This modification seems to make myr-PKC-α unable to inhibit PKC-β and thus makes the peptide selective to the PKC-α isozyme. In addition, the myristoylated peptide derived from the sequence of the endogenous inhibitor of cAMP-dependent protein kinase A, PKI, was used as a control for the sequence specificity of the inhibitory effect. PKI had no effect on DAG-induced contraction of either LES or ESO smooth muscle.

To conclude, PKC-dependent contraction of the circular smooth muscle of the LES is mediated by the calcium-dependent PKC-βII isozyme, whereas contraction of the circular smooth muscle of the ESO is mediated by the calcium-independent PKC-ϵ isozyme. Other PKC isozymes present in ESO and LES may mediate other functions.

References

1. Biancani, P., C. Hillemeier, K. N. Bitar, and G. M. Makhlouf. Contraction mediated by Ca^{2+} influx in esophageal muscle and by Ca^{2+} release in the LES. *Am. J. Physiol.* **253:**G760–G766 (1987).
2. Sohn, U. D., T. T. Chiu, K. N. Bitar, C. Hillemeier, J. Behar, and P. Biancani. Calcium requirements for ACh induced contraction of cat esophageal circular muscle cells. *Am. J. Physiol.* **266:**G330–G338 (1994).
3. Biancani, P., K. M. Harnett, U. D. Sohn, B. Y. Rhim, J. Behar, C. Hillemeier, and K. N. Bitar. Differential signal transduction pathways in LES tone and response to ACh. *Am. J. Physiol.* **266:**G767–G774 (1994).
4. Nishizuka, Y. Protein kinase C and lipid signaling for sustained responses. *FASEB J.* **9:**484–496 (1995).
5. Bitar, K. N., and G. M. Makhlouf. Receptors on smooth muscle cells: characterization by contraction and specific antagonists. *Am. J. Physiol.* **242:**G400–G407 (1982).
6. Biancani, P., M. Zabinski, M. Kerstein, and J. Behar. Lower esophageal sphincter mechanisms: anatomic and physiologic relationships of the esophagogastric junction of the cat. *Gastroenterology* **82:**468–475 (1982).
7. Bitar, K. N., S. Stein, and G. Omann. Specific G-proteins mediate endothelin induced contraction. *Life Sci.* **50:**2119–2124 (1992).
8. Sohn, U. D., K. M. Harnett, G. De Petris, J. Behar, and P. Biancani. Distinct muscarinic receptors, G proteins, and phospholipases in esophageal and lower esophageal sphincter (LES) circular muscle. *J. Pharmacol. Exp. Ther.* **267:**1205–1214 (1993).
9. Zoukhri, D., R. R. Hodges, C. Sergheraert, and D. Dartt. Lacrimal gland PKC isoforms are differentially involved in agonist-induced protein secretion. *Am. J. Physiol.*
10. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680–685 (1970).
11. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annal. Biochem.* **72:**248–254 (1976).
12. Hafner, D., E. Heinen, and E. Noack. Mathematical analysis of concentration-response relationships: method for the evaluation of the ED_{50} and the number of binding sites per receptor molecule using logit transformation. *Arzneim.-Forsch.* **27:**1871–1873, 1977.
13. Kraft, A. S., and W. B. Anderson. Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with the plasma membrane. *Nature (Lond.)* **301:**621–623 (1983).
14. Khalil, R. A., and K. G. Morgan. Imaging of protein kinase C distribution and translocation in living vascular smooth muscle cells. *Circ. Res.* **69:**1626–1631 (1991).
15. Haller, H., J. I. Smallwood, and H. Rasmussen. Protein kinase C translocation in intact vascular smooth muscle strips. *Biochem. J.* **270:**375–381 (1990).
16. Jiang, M. J., and K. G. Morgan. Intracellular calcium levels in phorbol ester-induced contractions of vascular muscle. *Am. J. Physiol.* **253:**H1365–H1371 (1987).
17. Jiang, M. J., and K. G. Morgan. Agonist-specific myosin phosphorylation and intracellular calcium during isometric contractions of arterial smooth muscle. *Pflueg. Arch. Eur. J. Physiol.* **413:**637–643 (1989).
18. Singer, H. A., and K. M. Baker. Calcium dependence of phorbol ester-induced force and myosin light chain phosphorylation in arterial smooth muscle. *J. Pharmacol. Exp. Ther.* **243:**814–821 (1987).
19. Hillemeier, C., K. N. Bitar, U. D. Sohn, and P. Biancani. Protein kinase C mediates spontaneous tone in the cat lower esophageal sphincter. *J. Pharmacol. Exp. Ther.* **277:**144–149 (1996).
20. Sohn, U. D., B. Han, A. H. Tashjian Jr., J. Behar, and P. Biancani. Agonist independent, muscle type specific signal transduction pathways in cat esophageal and lower esophageal sphincter (LES) circular smooth muscle. *J. Pharmacol. Exp. Ther.* **273**:482–491 (1995).
21. Sohn, U. D., D. K. Kim, J. V. Bonventre, J. Behar, and P. Biancani. Role of 100 kDa cytosolic PLA2 in ACh-induced contraction of esophageal circular muscle. *Am. J. Physiol.* **267:**G433–G441 (1994).
22. Nishizuka, Y. The family of protein kinase C for signal transduction. *JAMA* **262:**1826–1833 (1991).
23. Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Washington D. C.)* **258:**607–614 (1992).
24. Ono, Y., K. Fuji, U. Ogita, K. Kikkawa, K. Igarashi, and Y. Nishizuka. Protein kinase C subspecies from rat brain: its structure, expression, and properties. *Proc. Natl. Acad. Sci. USA* **86:**3099–3103 (1989).
25. Ali, S., M. W. Becker, M. G. Davis, and G. W. Dorn. Dissociation of vasoconstrictor-stimulated basic fibroblast growth factor expression from hypertrophic growth in cultured vascular smooth muscle cells: relevant roles of protein kinase C. *Circ. Res.* **75:**836–843 (1994).
26. Ignoguchi, T., R. Battan, E. Handler, J. R. Sportsman, W. Health, and G. I. King. Preferential elevation of protein kinase C isoform *beta* II and diacylglycerol levels in aorta and heart of diabetic rats: differential reversibility to glycemic control by island cell transplantation. *Proc. Natl. Acad. Sci. USA* **89:**11059–11063 (1992).
27. Khalil, R. A., C. Lajoie, M. S. Resnick, and K. G. Morgan. Ca^{2+}-independent isoforms of protein kinase C differentially translocate in smooth muscle. *Am. J. Physiol.* **263:**C714–C719 (1992).
28. Assender, J. W., E. Knotny, and B. B. Fredholm. Expression of protein kinase C isoforms in smooth muscle cells in various states of differentiation. *FEBS Lett.* **342:**76–80 (1994).
29. Dixon, B. S., R. V. Sharma, T. Dickerson, and J. Fortune. Bradykinin and angiotensin II: activation of protein kinase C in arterial smooth muscle. *Am. J. Physiol.* **266:**C1406–C1420 (1994).
30. Mochly-Rosen, D., H. Khaner, and J. Lopez. Identification of intracellular receptor proteins for activated protein kinase C. *Proc. Natl. Acad. Sci. USA* **88:**3997–4000 (1991).

31. Mochly-Rosen, D. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science (Washington D. C.)* **268:**247–250 (1995).
32. Baier, G., D. Telford, L. Giampa, K. M. Coggsshall, G. Baier-Bitterlich, N. Isakov, and A. Altman. Molecular cloning and characterization of PKCθ, a novel member of the protein kinase C (PKC) family expressed predominantly in hematopoietic cells. *J. Biol. Chem.* **268:**4997–5004 (1993).
33. Johannes, F.-J., J. Prestle, S. Eis, P. Oberhagamann, and K. Pfizenmaier. PKCμ is a novel, atypical member of the protein kinase C family. *J. Biol. Chem.* **269:**6140–6148 (1994).
34. Osadas, S., K. Mizuno, T. C. Saido, K. Suzuki, T. Kuroki, and S. Ohno. A new member of the protein kinase family, nPKCθ, predominantly expressed in skeletal muscle. *Mol. Cell. Biol.* **12:**3030–3938 (1992).
35. Flint, A. J., R. D. Paladini, and D. E. J. Koshland. Autophosphorylation of protein kinase C at separated regions of its primary sequence. *Science (Washington D. C.)* **249:**408–411 (1990).
36. Mitchell, F. E., R. M. Marias, and P. J. Parker. The phosphorylation of protein kinase C as a potential measure of activation. *Biochem. J.* **261:** 131–136 (1989).
37. Zhang, J., L. Wang, J. Schwartz, R. W. Bond, and W. R. Bishop. Phosphorylation of Thr 642 is an early event in the processing of newly synthesized protein kinase C *beta* I and is essential for its activation. *J. Biol. Chem.* **269:**19578–19584 (1994).
38. House, C., and B. E. Kemp. Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. *Science (Washington D. C.)* **238:**1726–1728 (1987).
39. Eichholtz, T., D. B. deBont, J. deWidt, R. M. J. Liskmp, H. L. Ploegh. A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. *J. Biol. Chem.* **268:**1982–1986 (1993).

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