MAPK mediates PKC-dependent contraction of cat esophageal and lower esophageal sphincter circular smooth muscle

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Cao, Weibiao, Uy Dong Sohn, Khalil N. Bitar, Jose Behar, Piero Biancani, and Karen M. Harnett. MAPK mediates PKC-dependent contraction of cat esophageal and lower esophageal sphincter circular smooth muscle. Am J Physiol Gastrointest Liver Physiol 285: G86–G95, 2003; 10.1152/ajpgi.00156.2002.—Esophageal (ESO) circular muscle contraction and lower esophageal sphincter (LES) tone are PKC dependent. Because MAPKs may be involved in PKC-dependent contraction, we examined ERK1/ERK2 and p38 MAPKs in ESO and LES. In permeabilized LES muscle cells, ERK1/2 antibodies reduced 1,2-dioctanoylglycerol (DG)- and threshold ACh-induced contraction, which are PKC dependent, but not maximal ACh, which is calmodulin dependent. LES tone was reduced by the ERK1/2 kinase inhibitor PD-98059 and by the p38 MAPK inhibitor SB-203580. In permeable ESO cells, ACh contraction was reduced by ERK1/ERK2 and p38 MAPK antibodies and by PD-98059 and SB-203580. ACh increased MAPK activity and phosphorylation of MAPK and of p38 MAPK. The 27-kDa heat shock protein (HSP27) antibodies reduced ACh contraction. HSP27 and p38 MAPK antibodies together caused no greater inhibition than either one alone. p38 MAPK and HSP27 coprecipitated after ACh stimulation, suggesting that HSP27 is linked to p38 MAPK. These data suggest that PKC-dependent contraction in ESO and LES is mediated by the following two distinct MAPK pathways: ERK1/2 and HSP27-linked p38 MAPK.

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The p38 MAPK is thought to be activated by inflammatory cytokines and environmental stress; it was identified as part of a protein kinase cascade activated by interleukin-1β or physiological stress and ending in activation of MAPK-activated protein (MAPKAP) kinase 2 and phosphorylation of the 25-27-kDa heat shock proteins HSP25/HSP27 (26, 50). MAPKAP kinase 2 phosphorylates HSP25/HSP27 in a cell-free preparation at the sites phosphorylated in intact cells in response to stress (56).

Phosphorylation of HSP27 has been described as an MAPK-mediated mechanism modulating contraction of intestinal (15) and vascular smooth muscle (45, 62). In rectosigmoid smooth muscle, p38 MAPK is activated during PKC-dependent contraction and cotranslocates with HSP27 (61).

In the current study, we examine the hypothesis that PKC-mediated contraction of LES and esophageal muscle depends on activation of MAPKs. We find that the PKC-dependent contractile pathway responsible for maintenance of LES resting tone and contraction in response to low concentrations of the endogenous neurotransmitter ACh depends on activation of MAPKs. Similarly, in esophageal smooth muscle, contraction in response to ACh depends on ERK MAPK phosphorylation and/or HSP27-linked p38 MAPK phosphorylation.

METHODS

Animals. Adult male cats weighing between 3.5 and 5.5 kg were initially anesthetized with ketamine (Aveco, Fort Dodge, IA) and then were killed with an overdose of pentobarbital sodium (Shering, Kenilworth, NJ). The chest and abdomen were opened with a midline incision exposing the esophagus and stomach. The esophagus and LES were isolated and excised as previously described (11, 13).

Measurements of in vitro LES tone. LES strips (2 mm) were mounted in separate 1-ml muscle chambers and equilibrated for 2 h with continuous perfusion of oxygenated physiological salt solution (PSS), as previously described in detail (9–13, 32). During this time, the tension in LES strips increased, attaining a steady level at 2 h. The PSS contained the following (in mM): 116.6 NaCl, 2.1 NaHCO3, 1.2 NaH2PO4, 3.4 KCl, 2.5 CaCl2, 5.4 glucose, and 1.2 MgCl2. The solution was equilibrated with a gas mixture containing 95% O2-5% CO2 at pH 7.4 and 37°C. After equilibration, LES strips were incubated for 30 min in solution containing vehicle (control) or the appropriate concentrations of PD-98059 and SB-203580. The vehicle for PD-98059 and SB-203580 was ethanol, which has no effect on strips at concentrations <0.1%. The highest concentration used in this study was 0.05%.

Smooth muscle tension was recorded on a chart recorder (Grass Instruments, Quincy, MA). Passive force was obtained at the end of the experiment by completely relaxing the strips with excess EDTA until no further decrease in resting force was observed. Basal LES tone is the difference between resting and passive force. Percent increase in basal tone was defined by the ratio between the increase in force after drug administration and basal LES tone. Percent basal LES tone was calculated by the ratio between the force after using the drugs and the basal LES tone.

Preparation of circular smooth muscle tissue. The esophagus and LES were excised, the circular muscle layer was cut into 0.5-mm-thick slices with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA), and tissue squares were made by cutting two times with a 2-mm blade block, the second cut at right angles to the first. This circular smooth muscle tissue was used for Western blot analysis of MAPK, measurement of MAPK activity and MAPK phosphorylation, and to obtain isolated smooth muscle cells.

Cell isolation and permeabilization. Isolated smooth muscle cells were obtained by enzymatic digestion, as previously described (9–12). Briefly, esophageal and LES circular smooth muscle was digested in HEPES-buffered physiological solution containing 150 μM collagenase (CLS type II; Worthington Biochemicals, Freehold, NJ) for 2 h. The HEPES solution contained 114.7 mM NaCl, 5.7 mM KCl, 2.1 mM KH2PO4, 11 mM glucose, 24.5 mM HEPES, 1.9 mM CaCl2, 0.57 mM MgCl2, 0.3 mg/ml BME amino acid supplement (M. A. Bioproducts, Walkersville, MD), and 0.08 mg/ml soybean trypsin inhibitor (Worthington Biochemicals). The HEPES solution was oxygenated (100% O2) at 31°C, and the pH was adjusted to 7.4. At the end of the digestion period, the tissue was rinsed and then incubated in collagenase-free HEPES buffer. The cells dissociate freely in collagenase-free solution.

When permeable cells are required to allow the use of MAPK and HSP antibodies that do not diffuse across the intact plasma membrane, the partly digested muscle tissue is washed with a “cytotoxic” enzyme-free PSS (cytotoxic buffer) of the following composition (in mM): 50 NaCl, 100 KCl, 25 NaHCO3, 5.0 MgSO4, 0.96 NaH2PO4, 1.0 EGTA, and 0.48 CaCl2. The cytotoxic buffer contained 2% BSA and was equilibrated with 95% O2-5% CO2 to maintain pH of 7.2 at 31°C. Muscle cells dispersed spontaneously in this medium. A low concentration of calcium is present in the cytotoxic buffer to avoid spontaneous contraction of the cells in the absence of agonists after the membrane becomes permeable. The cells are permeabilized by incubation for 3 min in cytotoxic buffer containing saponin (75 μg/ml). After exposure to saponin, the cell suspension is spun at low gravity, and the resulting pellet is resuspended in saponin-free modified cytotoxic buffer containing antimycin (10 μM), ATP (1.5 mM), and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphate kinase (10 U/ml; see Ref. 14).

Agonist-induced contraction of isolated muscle cells. Once the cells had dissociated, 0.5-ml aliquots of the cell-containing fluid were added to tubes for exposure to agonists and measurement of contraction. The maximally effective concentration of ACh for contraction of intact or saponin-permeabilized single cells is 10–10 to 10–9 M and lower than the dose required for a maximal response in undigested muscle tissue strips or squares (10–5 M; see Ref. 11) that were used for Western blots or for measurements of kinase activity. Intact esophageal circular smooth muscle cells were contracted with a maximally effective concentration of ACh alone or after 10 min of exposure to the indicated concentration of PD-98059 or SB-203580. The vehicle for PD-98059 and SB-203580 was ethanol, which has no effect on strips or cells at concentrations <0.1%. The highest concentration used in this study was 0.05%.

Permeabilized esophageal and LES cells were exposed to a maximally effective concentration of ACh or to the diacylglycerol analog 1,2-dioctanoylglycerol (DG, 10–6 M) for 30 s. When MAPK or HSP antibodies were used, permeabilized cells were incubated in the antiserum at the indicated concentration for 1 h before the addition of agonist (15). We have previously shown that specific steps in the signaling pathway can be selectively inhibited by antibodies against the appropriate proteins mediating the specific reaction (18, 52).
After exposure to agonist, the cells were fixed in acrolein at a final 1.0% concentration and kept refrigerated.

**Cell measurements.** A drop of the cell-containing medium was placed on a glass slide and covered by a coverslip. Thirty consecutive cells from each slide were observed through a phase-contrast microscope (Carl Zeiss) and a CCTV camera (model WV-CD51; Panasonic, Secaucus, NJ) connected to a Macintosh Computer (Apple, Cupertino, CA). The Image 1.59 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. The average cell length is the same between intact cells (66.9 ± 1.8 μm) and permeable cells (66.9 ± 3.8 μm). In addition, average cell length was measured after the addition of test agents. Shortening was defined as a percentage decrease in average length after agonists when compared with control length.

**Western blot.** Esophageal and LES circular muscle was homogenized in Trition X lysis buffer containing 50 mM Tris·HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (vol/vol) Triton X, 40 mM β-glycerolphosphate, 40 mM DTT, and 200 μM MAPK immunoprecipitation buffer. Esophageal circular muscle, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The suspension was centrifuged at 15,000 g for 5 min, and the protein concentration in the supernatant was determined. The supernatant of each sample containing the same amount of protein (150 μg ERK1/ERK2, 120 μg for p38 MAPK) was mixed with 2% SDS loading buffer containing 62.5 mM Tris·HCl (pH 6.8), 5% 1-mercaptoethanol, 0.002% bromphenol blue, and 10% glycerol and boiled for 5 min. Prestained molecular weight marker was prepared in the same manner. After these supernatant samples were subjected to SDS-PAGE (200 volts, 45 min) using an acrylamide concentration of 10% in the running gel and 4% in the separating gel for p38, ERK1, and ERK2 MAPK and 15% for HSP 27, the separated proteins were electrotransferred to a nitrocellulose (NC) membrane (Bio-Rad, Melville, NY) at 30 volts overnight. Transfer of proteins to the NC membrane was confirmed with Ponseau S staining reagent (Sigma, St. Louis, MO). To block non-specific binding, the NC membrane was incubated in 5% nonfat dry milk in PBS for 60 min followed by three rinses in milk-free buffer. Samples were incubated with anti-phosphorylated MAPK antibody (1:20,000; Promega, Madison, WI), anti-phosphorylated p38 MAPK antibody (1:300; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-HSP 27 antibody (1:100; Upstate Biotechnology, Waltham, MA) for 1 h 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 (Amersham, Arlington Heights, IL). Detection was achieved with an enhanced chemiluminescence agent (Amersham). Molecular weight was estimated by comparison of sample bands with prestained molecular weight marker (Amersham). For the p38 and ERK1/ERK2 MAPK phosphorylation, after detecting the phosphorylated MAPKs, the membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.6 mM Tris·HCl, pH 6.7) at 50°C for 30 min, washed three times (10 min each), and then reprobed by using anti-p38 MAPK antibody (1:500; Santa Cruz Biotechnology) and anti-ERK2 antibody (1:500; Santa Cruz Biotechnology), respectively.

**MAPK in esophageal and LES smooth muscle.** The MAPKs ERK1 (p44) and ERK2 (p42) were identifiable by Western blot analysis in esophageal and LES circular smooth muscle (Fig. 1). To test whether ERK1/ERK2 participate in PKC-dependent contraction, the cells were permeabilized with saponin to allow diffusion of antibodies in the cytoplasm and then contracted with the PKC activator DG. ERK1 and ERK2 antibodies concentration dependently inhibited the DG-induced contraction of LES and esophageal muscle (P = 0.001, ANOVA; Fig. 1), supporting the view that MAPKs play a role in PKC-dependent contractile pathways. The ERK2 antibody was more effective and at a 10 μg/ml concentration reduced shortening in response.
to DG from 20.0 ± 0.2 to 7.1 ± 0.6% in LES and from 21.5 ± 0.6 to 9.0 ± 0.7% in esophageal cells.

**LES tone and response to ACh.** We have previously shown that high and low ACh concentrations activate different signal transduction pathways in LES muscle. Low ACh activates a PKC-dependent contractile pathway, whereas high ACh concentrations are mediated through a calmodulin- and MLCK-dependent pathway (10, 51). To test whether inhibition of MAPKs is effective only against PKC-mediated contraction, LES cells were permeabilized with saponin, to allow diffusion of antibodies into the cytoplasm, and exposed to antibodies against ERK1 and ERK2. At low ACh concentration, when contraction is PKC dependent, the contraction was inhibited by the antibodies (Fig. 2). At high, (i.e., maximally effective) ACh concentration, when contraction is calmodulin- and MLCK-dependent and not dependent on PKC, the contraction was not affected, suggesting that ERK1 and ERK2 participate only in the PKC-dependent contractile pathway.

If PKC-dependent contraction is mediated by MAPKs, LES circular muscle resting tone, which is also mediated by a PKC-dependent contractile pathway (32), should depend on MAPKs. To test whether MAPKs play a role in maintenance of LES tone, LES circular muscle strips were mounted in a muscle chamber and allowed to equilibrate and develop spontaneous tonic contraction. After a steady tone had developed, the strips were exposed to increasing concentrations of inhibitors of MAPKs. The ERK1 and ERK2 MAPKs are activated by a MAP kinase kinase (MEK), which phosphorylates ERK1 and ERK2 on two sites. The selective MEK inhibitor PD-98059 and the p38 MAPK inhibitor SB-203580 concentration dependently reduced (P = 0.001, ANOVA) LES tone (Fig. 3) when used alone. LES tone was reduced further when PD-98059 and SB-203580 were used in combination (P = 0.001, ANOVA). These data suggest the involvement of ERK1, ERK2, and p38 MAPKs in PKC-dependent LES tone and suggest that MAPKs may participate in PKC-dependent muscle contractions. To confirm that PKC-dependent contraction is mediated by ERK1/2 and p38 MAPK, LES cells were contracted with the diacylglycerol analog DG, which directly activates PKC. Similarly to spontaneous tone, DG-induced contraction of LES cells was reduced by PD-98059 and by SB-203580 (P = 0.001, ANOVA) and was further reduced when PD-98059 and SB-203580 were used in combination (P = 0.001, ANOVA; Fig. 4)

**Esophageal contraction.** Because contraction of esophageal muscle in response to its physiological neu-
MAPKs mediated through activation of both ERKs and p38 may be entirely (P < 0.001, ANOVA). PD-98059 and SB-203580 in combination nearly abolished DG-induced contraction of LES cells (P = 0.001, ANOVA). Values are means ± SE of 3 animals with 30 cells counted for each animal.

The selective MEK inhibitor PD-98059 concentration-dependently reduced ACh-induced contraction (P < 0.001, ANOVA) of intact esophageal smooth muscle cells in response to ACh. A maximally effective concentration of PD-98059 reduced ACh-induced contraction from 22.9 ± 1.4 to 9.7 ± 0.8%. Similarly, the selective p38 MAPK inhibitor SB-203580 concentration dependently reduced shortening of intact esophageal cells in response to a maximally effective concentration of ACh (10⁻⁹ M; Fig. 5; P < 0.001, ANOVA). A maximally effective concentration of SB-203580 reduced ACh-induced contraction from 23.9 ± 1.4 to 14.7 ± 0.4%

SB-203580 and PD-98059, when used in combination, nearly abolished contraction of esophageal muscle (P < 0.01, ANOVA), supporting the view that esophageal contraction in response to ACh may be entirely mediated through activation of both ERKs and p38 MAPKs.

To confirm the contribution of ERK1, ERK2, and p38 MAPKs to ACh-induced contraction, esophageal smooth muscle cells were permeabilized and then contracted with a maximally effective concentration of ACh (10⁻⁹ M) in the presence of ERK1, ERK2, and p38 MAPK antibodies (Fig. 6). ACh-induced contraction was concentration dependently reduced (P = 0.001, ANOVA) by ERK1 and ERK2 antibodies. ERK2 antibodies caused a greater reduction in contraction than ERK1. ERK2 antibodies (10 μg/ml) reduced shortening in response to ACh from 23.3 ± 0.6 to 8.4 ± 0.1%. The reduction by the ERK2 antibody was similar to that produced by the MEK inhibitor PD-98059 at a maximally effective concentration (from 22.9 ± 1.4 to 9.7 ± 0.8%). Similarly, ACh-induced contraction was concentration dependently reduced (P = 0.001, ANOVA) by a p38 MAPK antibody (Fig. 6). The reduction produced by the p38 antibody at its maximally effective concentration (from 25.5 ± 0.3 to 13.4 ± 0.7) was similar to that produced by the p38 MAPK inhibitor SB-203580 (23.9 ± 1.4 to 14.7 ± 0.4%).

Agonist-induced phosphorylation of HSP27 has been proposed as an MAPK-mediated mechanism of smooth muscle contraction (8, 15, 17, 43, 61). We therefore examined the role of HSP27 on esophageal contraction. HSP27 antibodies concentration dependently reduced contraction in response to a maximally effective concentration of ACh (10⁻⁹ M; P < 0.05, ANOVA; Fig. 7). HSP27 and p38 MAPK antibodies in combination caused no greater inhibition than either one alone (Fig. 7), suggesting that p38 MAPK and HSP27 act in the same signal transduction pathway.

MAPK activity and phosphorylation. To confirm that ACh-induced contraction of esophageal circular smooth muscle results from MAPK activation, we measured MAPK activity and phosphorylation. MAPK was purified by immunoprecipitation from esophageal circular muscle after incubation with 10⁻⁵ M ACh for 0, 30, 60, and 300 s. MAPK activity (ERK2) significantly increased from 0.12 ± 0.04 pmol·min⁻¹·mg protein⁻¹ at the basal level to 0.31 ± 0.06 and 0.34 ± 0.06 pmol·min⁻¹·mg protein⁻¹ (P < 0.05) at 30 and 60 s after ACh stimulation (Fig. 8).
Phosphorylation of ERK MAPK in response to ACh was measured by Western blot analysis using activated (phosphorylated) ERK2 antibodies (Fig. 9). Figure 9 shows that ERK2 phosphorylation of esophageal circular smooth muscle increased significantly after 1 min of stimulation with ACh (10⁻⁵ M; P < 0.01), confirming that ACh-induced contraction of esophageal circular smooth muscle is associated with MAPK activation. In addition, the PKC inhibitor chelerythrine (10⁻⁵ M), abolished the ACh-induced increase in ERK2 phosphorylation of esophageal circular smooth muscle, demonstrating that activation of ERK2 is PKC dependent.

Phosphorylation of p38 MAPK in response to ACh was also measured by Western blot analysis using activated (i.e., phosphorylated) p38 MAPK antibodies (Fig. 10). Phosphorylation of p38 MAPK in esophageal circular smooth muscle significantly increased to 604 ± 169% of control after 15–60 s stimulation with ACh (10⁻⁵ M; P < 0.05) and decreased at 300 s, demonstrating that ACh-induced contraction of esophageal circular smooth is associated with activation of p38 MAPK.

To confirm that p38 MAPK and HSP27 act in the same signal transduction pathway, esophageal tissue squares were stimulated with ACh and then homogenized and immunoprecipitated with a phosphorylated p38 MAP antibody. The immunoprecipitate was then probed with an HSP27 antibody. The immunoprecipitate contained increased levels of HSP27 after stimulation with ACh compared with unstimulated muscle (Fig. 11). We interpret these findings to mean that ACh stimulation and consequent activation of p38 MAPK promote association of HSP27 with the phosphorylated (i.e., active) p38 MAPK. This interpretation is further supported by the finding that the time-dependent association of HSP27 and phosphorylated p38 MAPK...
Fig. 11. ESO tissue squares were stimulated with ACh (10^{-5} M) and then homogenized and immunoprecipitated with a phosphorylated p38 MAPK antibody. HSP27 levels were then detected in the immunoprecipitate. A: Western blot of the immunoprecipitate with an HSP27 antibody found greater amounts of HSP27 at 15, 30, and 60 s (P < 0.05) after stimulation with ACh than in unstimulated muscle. Values are means ± SE of 3 animals. B: typical Western blot. The data suggest that HSP27 is linked to phosphorylated p38 MAPK after ACh stimulation.

**DISCUSSION**

Contraction of esophageal circular muscle in response to its endogenous neurotransmitter ACh is mediated by PKC-ε (53). A PKC (PKC-δ)-dependent contractile pathway is also present in LES circular muscle, where it mediates maintenance of LES resting tone and contraction in response to a low level of ACh (10, 32), whereas LES contraction in response to a maximally effective concentration of ACh is mediated by a calmodulin-dependent pathway (10, 51). In the current study, our data suggest that MAPK activation participates only in these PKC-dependent contractions of LES and esophageal circular smooth muscle and not in calmodulin-dependent LES contraction in response to maximally effective concentrations of ACh.

To demonstrate that MAPK participates in PKC-mediated contraction of esophageal and LES circular muscle, we tested for the presence of ERK1 and ERK2 by Western blot analysis. ERK1 and ERK2 are present in LES and esophageal circular smooth muscle, consistently with numerous studies that report expression of MAPKs in numerous cell types. In addition, when contraction was induced by the diacylglycerol analog DG, ERK1 and ERK2 antibodies concentration dependently reduced contraction in both esophageal circular muscle and LES. Similarly to diacylglycerol, DG is known to directly activate PKC, confirming that MAPK participates in PKC-mediated contraction of esophageal and LES circular muscle.

We next examined in detail the participation of MAPKs in contraction of LES and esophageal muscle.

**MAPK and LES smooth muscle contraction.** In LES circular muscle, we therefore tested the role of MAPKs in the response to high and low concentrations of ACh and in LES spontaneous tone. Our data show that inhibition of MAPKs by selective antibodies inhibits contraction induced by a low concentration ACh and by DG, both of which activate a PKC-dependent, calmodulin-independent contractile pathway. The antibodies had no effect on contraction induced by a high (i.e., maximally effective) concentration of ACh, which is mediated by calmodulin. Similarly, LES tone, which is PKC dependent, was concentration dependently reduced by the MEK inhibitor PD-98059 and by the p38 MAPK inhibitor SB-203580 when used alone. When used in combination, the two inhibitors further reduced tone. The same MAPK inhibitors, when used alone, reduced DG-induced contraction of intact LES cells and, in combination, almost abolished it.

PD-098059 has been reported to selectively inhibit the MAPK-activating enzyme MAPK/ERK (MEK) without inhibiting activity of MAPK itself. Inhibition of MEK by PD-098059 prevents activation of MAPK and subsequent phosphorylation of MAPK substrates both in vitro and in intact cells (4, 23). SB-203580 is a pyridinyl imidazole derivative that selectively inhibits the kinase activity of p38 (44). The effectiveness and selectivity of inhibitors and antibodies are supported by the similarity in the results.

In addition, selectivity of the antibodies is supported by the fact that ERK1/2 antibodies inhibited contraction of permeable LES cells by a low concentration of ACh but not contraction induced by a high concentration of ACh. These findings are consistent with numerous studies (18) showing that antibodies against particular proteins in the contractile pathway are as effective as selective inhibitors of the same protein. This finding may be because of the relatively high molecular weight of immunoglobulins. It is entirely possible that, once antibodies bind to a protein, they may inhibit its effect because of their size, regardless of their particular binding site on the protein.

The data suggest that in LES circular muscle MAPKs (ERKs and p38) may participate in PKC-dependent contractions, such as LES tone or contraction in response to the PKC activator DG, or in response to low concentrations of ACh. Contraction in response to a maximally effective concentration of ACh, which is calmodulin dependent, was not affected by MAPK antibodies, confirming MAPK involvement only in PKC-mediated contraction.

**MAPK and esophageal smooth muscle contraction.** Similarly to spontaneous LES tone, contraction of esophageal muscle in response to its endogenous neurotransmitter ACh is PKC dependent (10, 32, 53) and therefore may depend on activation of MAPKs. As expected, either inhibition of MEK, the upstream activator of MAPK, by PD-980959 or inhibition of p38 kinase by SB-203580 reduced ACh-induced contraction of esophageal smooth muscle when used alone. In combination, SB-203580- and PD-980959-induced inhibition was additive, resulting in almost complete abolition of ACh-induced contraction. These results with MAPK inhibitors were confirmed in saponin-permeabilized cells with ERK1, ERK2, and p38 kinase antibodies. Similarly to the inhibitors, ERK or p38 kinase
antibodies dose dependently reduced ACh-induced contraction of esophageal cells. The effectiveness and selectivity of inhibitors and antibodies is supported by the similarity in the results.

Involvement of MAPKs in PKC-mediated ACh-induced contraction of esophageal muscle is confirmed by MAPK activity measurements that show increased MAPK activity in response to ACh and abolition of ERK2 phosphorylation by the PKC inhibitor chelerythrine. These results are consistent with other studies reporting MAPK activation by a variety of contractile agonists in vascular, airway, and intestinal smooth muscles (27, 28, 34). For example, in tracheal smooth muscle cells, ACh stimulates ERK1 and ERK2 phosphorylation, which is significantly reduced by PD-98059 (31).

In the current study, our data indicate that PKC-dependent contraction is associated with activation of MAPK. A connection between MAPK activation and PKC-mediated smooth muscle contraction has been proposed (36, 37) for ferret aorta, where MAPK is activated in response to phenylephrine (46). In this preparation, PKC-ε and ERK1 MAPK translocate from the cytosol to the sarcolemma in response to adrenergic stimuli; PKC-ε remains associated with the sarcolemma, whereas MAPK redistributes to the cytosol coincident with contraction (38, 39). There is some confusion, however, about the role of MAPK activation in smooth muscle contraction. In swine carotid artery, agonists such as histamine and direct PKC activation by phorbol 12,13-dibutyrate (PDBu) induced MAPK activation and phosphorylation, but inhibition of the MAPK cascade by PD-098059 did not affect histamine or PDBu-induced contraction (30). In tracheal smooth muscle, M2 receptor activation of ERK MAPKs and phosphorylation had little or no effect on isometric force (31). It has been proposed that these conflicting reports may be because of tissue-specific differences related to the calcium dependence of the particular contraction. Calcium-independent contractions of smooth muscle may be more dependent on MAPK-mediated pathways than calcium-dependent contraction. For example, the swine carotid artery does not contract in response to agonist activation in the absence of extracellular calcium (30). However, calcium-independent contractions induced by phenylephrine in the ferret aorta (22), histamine in bovine tracheal smooth muscle (41), and PGF2α in the iris sphincter smooth muscle (63) have all been significantly reduced by PD-98059.

No such confusion exists for contraction of LES and esophageal smooth muscle. In these tissues, there is a clear distinction between calcium-calmodulin-MLCK-dependent contraction and PKC-dependent contraction, which is not dependent on activation of the calcium-sensitive MLCK. The contractile paradigm for the calcium-calmodulin-MLCK-dependent contraction has been understood for some time. Understanding of the mechanisms responsible for the PKC-dependent contractile pathways is still evolving, and considerable confusion is derived from attempts to explain PKC-dependent contractile pathways as variants of the MLCK-dependent pathway, involving increased sensitization to calcium, or other mechanisms (29, 40, 54, 55). In the esophagus and LES, the two pathways are clearly distinct and independent of each other. Even in the LES, where both pathways exist at low calcium levels, which are insufficient to fully activate calmodulin, contraction is PKC dependent, whereas when calcium levels are sufficient to fully activate calmodulin, the activated calmodulin inhibits PKC and activates MLCK. In both tissues, ERK1, ERK2, and p38 MAPKs participate only in the PKC-dependent contractile pathway and not in the calmodulin- and MLCK-dependent pathway. In addition, the finding that combined inhibition of the ERK1, ERK2, and p38 MAPKs results in almost complete inhibition of LES tone and DG contraction, and of esophageal contraction, supports the view that PKC-dependent contraction of these muscles is almost entirely mediated through activation of these MAPKs.

In addition to a role of the ERK MAPKs, our data demonstrate a role of the p38 MAPK in PKC-mediated contraction. The contractile pathway mediated by p38 MAPK may be linked to HSP27 because HSP27 and p38 MAPK antibodies in combination caused no greater inhibition of contraction than either one alone. In a different smooth muscle preparation, it has been proposed that p38 MAPK may be activated during PKC-dependent contraction and cotranslocated with HSP27 (61). HSP27 is an actin filament-binding protein (47) that may contribute to PKC-dependent contraction in intestinal smooth muscle (61). Phosphorylation of HSP27 increases after stimulation with contractile agonists such as carbachol (43), thrombin (17), C2-ceramide and endothelin-1 (17), and cyclosporin A (8). Smooth muscle contraction is reduced by inhibition of HSP27 phosphorylation (8, 15) or by changes in the intracellular distribution of HSP27 (59).

To confirm the link between HSP27 and p38 MAPK, esophageal muscle was stimulated with ACh, then homogenized and immunoprecipitated with p38 antibody. Muscle homogenates, immunoprecipitated with a phosphorylated p38 MAPK antibody, contained increased levels of HSP27 after stimulation with ACh compared with unstimulated muscle, suggesting that ACh stimulation and consequent activation of p38 MAPK promote association of HSP27 with the phosphorylated (i.e., active) p38 MAPK. This interpretation is further supported by the finding that the time-dependent association of HSP27 and phosphorylated p38 MAPK (Fig. 11) closely mirrors the time course of p38 MAPK phosphorylation (Fig. 10). These results are consistent with reports of ANG II-induced contraction of rat vascular smooth muscle, where p38 MAPK-dependent phosphorylation of HSP27 contributes to contraction (45).

Our data suggest that contraction of esophageal circular smooth muscle is mediated by ERKs and p38 MAPKs, consistent with the finding that contraction is almost abolished by a combination of ERK and p38 MAPK inhibitors.
In addition, the actin-binding proteins calponin and caldesmon may be involved in PKC- and MAPK-dependent contraction (51). Calponin and caldesmon bind to actin and inhibit the Mg²⁺-ATPase of phosphorylated smooth muscle myosin, thereby preventing cross-bridge cycling and smooth muscle contraction. Actin binding and ATPase inhibition are abolished by phosphorylation of these thin-filament proteins by PKC and calcium- and calmodulin-dependent protein kinase II and restored by dephosphorylation (5). Caldesmon may mediate MAPK-dependent contraction because it is phosphorylated during contraction (3), the phosphorylation sites on caldesmon have been identified as MAPK sites (2), and a caldesmon inhibitory peptide contracts permeable smooth muscle cells (35, 51). Calponin may function as an adapter protein connecting the PKC cascade to the ERK cascade because it coprecipitates with ERK1 and PKC in ferret aorta homogenates, colocalizes in cells with ERK1 and PKC, and binds activated PKC in a gel overlay assay (46). We have reported that, in permeable cells of the esophagus, calponin and caldesmon play a role in MLCK-independent, PKC-dependent contraction. Both calponin and caldesmon inhibit DG-induced (i.e., PKC-dependent) contraction of esophageal muscle but do not affect LES contraction in response to a maximally effective concentration of ACh, which is calmodulin dependent (51).

Activation of the PKC contractile pathway may result in phosphorylation of MEK, which in turn may phosphorylate MAPK. MAPK may then phosphorylate either calponin or caldesmon or some intermediate protein, resulting in caldesmon/calponin phosphorylation (2). Caldesmon and calponin, when phosphorylated, change conformation and no longer inhibit actomyosin interaction, allowing contraction to occur. It is possible that two distinct parallel pathways may contribute to PKC-dependent contraction, one involving p38 kinase and HSP25/27 and the other one involving ERKs and calponin/caldesmon.

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