Myosin light chain kinase- and PKC-dependent contraction of LES and esophageal smooth muscle

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In smooth muscle cells enzymatically isolated from circular muscle of the esophagus (ESO) and lower esophageal sphincter (LES), ACh-induced contraction and myosin light chain (MLC) phosphorylation were similar. Contraction and phosphorylation induced by purified MLC kinase (MLCK) were significantly greater in LES than ESO. ACh-induced contraction and MLC phosphorylation were inhibited by calmodulin and MLCK inhibitors in LES and by protein kinase C (PKC) inhibitors in ESO. Contraction of LES and ESO induced by the PKC agonist 1,2-dioctanoylglycerol (DG) was unaffected by MLCK inhibitors. Caldesmon and calponin concentration-dependently inhibited ACh-induced contraction of ESO and not LES. In ESO, caldesmon antagonist GS17C reversed caldesmon- but not calponin-mediated contraction of permeabilized ESO but had much less effect on LES. GS17C-induced contraction was not affected by MLCK inhibitors, suggesting that MLCK may not regulate caldesmon-mediated contraction. DG-induced contraction of ESO and LES was inhibited by caldesmon and calponin, suggesting that these proteins may regulate PKC-dependent contraction. We conclude that calmodulin and MLCK play a role in ACh-induced LES contraction, whereas the classical MLCK may not be the major kinase responsible for contraction and phosphorylation of MLC in ESO. ESO contraction is PKC dependent. Caldesmon and/or calponin may play a role in PKC-dependent contraction.

calcium stores; caldesmon; calmodulin; calponin; cat; second messenger system
proteins caldesmon and calponin in contraction of LES and ESO. We find that in ESO, contraction in response to agonists or direct PKC activation by 1,2-dioctanoylglycerol (DG) is calmodulin independent and not regulated by MLCK, even though ACh-induced contraction of ESO is associated with MLC phosphorylation. In contrast to LES, activation by a maximally effective concentration of ACh results in calmodulin- and MLCK-dependent contraction. However, LES contraction in response to direct PKC activation by DG is similar to contraction of ESO and is calmodulin independent and not affected by MLCK inhibitors. In addition, caldesmon and calponin do not play a role in MLCK-dependent contraction of LES but may exert a regulatory effect on PKC-dependent contraction of ESO and LES.

We conclude that in these two types of muscle there are two distinct types of contractions that depend on distinct regulatory mechanisms. A PKC-dependent pathway, which may be regulated by caldesmon and calponin, is present in ESO and LES. A calmodulin and MLCK-dependent pathway is present only in LES muscle. MLC phosphorylation occurs in both pathways.

METHODS

Animals. Adult cats of either sex, weighing between 2.5 and 5 kg, were used. The animals were initially anesthetized with ketamine (Aveco, Fort Dodge, IA) then euthanized with an overdose of phenobarbital (Schering, Kenilworth, NJ). The chest and abdomen were opened with a mid-line incision exposing the ESO and stomach. The ESO and stomach were removed together, opened along the lesser curvature, and pinned on a wax block at their in vivo dimensions. The location of the squamocolumnar junction was identified, and the mucosa was peeled. The high-pressure zone of the LES is characterized by a visible thickening of the circular muscle layer in correspondence to the squamocolumnar junction and immediately proximal to the fimbriae of the stomach.

Preparation of tissue squares. After opening the ESO and stomach and identifying the LES, we removed the mucosa and submucosal connective tissue by sharp dissection. The LES was excised, and the circular muscle layer 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. The slices of circular muscle were placed flat on a wax surface, and tissue squares were made by cutting twice with a 2-mm blade block, with the second cut at right angles to the first. Tissue squares were used in the enzymatic digestion process, the partly digested tissue (see Figs. 4 and 5) and permeabilized circular muscle. MLC phosphorylation occurs in both pathways.

Preparation of permeable smooth muscle cells. Cells were permeabilized to control cytosolic calcium concentration and to allow diffusion of calmodulin, MLCK, caldesmon, and calponin across the cell membrane. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with a cytosolic enzyme-free medium (cytosolic buffer) of the following composition (in mM): 20 NaCl, 100 KCl, 25 NaHCO₃, 5 MgSO₄, 0.96 NaH₂PO₄, 1 EGTA, and 0.48 CaCl₂. The medium contained 2% BSA and was equilibrated with 95% O₂-5%CO₂ to maintain a pH of 7.2 at 31°C. Muscle cells dispersed spontaneously in this medium.

Isolated cells were permeabilized by a 3-min incubation in cytosolic buffer containing saponin (75 μg/ml). Permeabilized cells were washed in saponin-free cytosolic buffer containing 0.5 mM CaCl₂ and 1 mM EGTA, yielding 180 nM free calcium (as described previously by Fabiato and Fabiato [19]).

When different calcium concentrations were required, calcium concentrations were changed as needed, and free calcium levels were similarly calculated. The modified cytosolic buffer also contained antimycin (10 μM), ATP (ATP disodium salt, 1.5 mM), and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase (10 U/ml) [11].

Agonist-induced contraction of isolated muscle cells. Cells were contracted by 30-s exposure to ACh, DG, GS17C, or MLCK. The concentration of ACh required to produce a maximal contraction of isolated muscle cells is 10⁻¹⁰–10⁻⁹ M (see RESULTS). When inhibitors were used, the cells were incubated in their presence for 1 min before addition of agonists. Cells were equilibrated in each calcium concentration for several minutes during the permeabilization process and before calmodulin addition. When MLCK was used, cells were incubated in threshold concentrations of calcium and calmodulin. At the appropriate time interval, cells were fixed in acrolein at a final 1% concentration.

The length of 30 consecutive intact cells from each slide was measured through a phase-contrast microscope (Carl Zeiss) and a digital charge-coupled television camera (model WV CD-51, Panasonic, Secaucus, NJ). The camera was connected to a Macintosh IIci computer (Apple, Cupertino, CA). A software program, Image 1.59 (National Institutes of Health, Bethesda, MD), was used to obtain a measurement of cell length. Intact or viable cells are distinguished by a membrane that is bright, smooth, and shiny, with the appearance of a halo around the periphery of the cell. These cells will contract when placed on a shallow muscle chamber under an inverted microscope and “spritzed” with ACh using a pressure ejection micropipette system [13]. Data were expressed as cell shortening defined as percent decrease in cell length from control.

Determination of myosin phosphorylation. For measurement of myosin phosphorylation, intact circular smooth muscle tissue (see Figs. 4 and 5) and permeabilized circular smooth muscle cells (see Fig. 6) were used. For ACh-induced myosin phosphorylation, tissue samples were equilibrated in oxygenated Krebs PSS at 37°C for 2 h and then exposed to a concentration of ACh (10⁻⁵ M) used to obtain a maximal contractile response. We (8) previously found that this is the maximally effective ACh concentration in intact circular muscle. The reaction was stopped by freezing the samples in a slurry of acetone and dry ice, after 7 s for ESO and 10 s for LES. These are the times required for these muscles to
achieve two-thirds of maximal contraction in response to ACh (8).

For MLCK-induced phosphorylation, permeabilized smooth muscle cells were equilibrated in the indicated concentration of calcium and calmodulin for 30 min. The concentration of calcium and calmodulin used produces a maximal contractile response (9, 70). Permeabilized smooth muscle cells were stimulated with the indicated concentration of MLCK for 30 s, and the reaction was stopped by freezing the samples in a slurry of acetone and dry ice.

Nonphosphorylated and phosphorylated forms of MLC were separated by electrophoresis and localized with antibodies against MLC. The relative amounts of phosphorylated and nonphosphorylated MLC were quantitated by densitometry (15, 41). Briefly, protein was extracted in an 8 M urea buffer and processed for urea/glycerol-PAGE as described previously by Persechini et al. (64). Nonphosphorylated and phosphorylated forms of the light chain were separated after electrophoresis at 20 °C and 400 V for 12–18 h. Proteins were electrophoretically transferred from glycerol gels onto nitrocellulose paper. MLC were localized on nitrocellulose paper with antibodies against MLC. Relative amounts of phosphorylated and nonphosphorylated MLC were quantitated from densitometry scans of the immunostained nitrocellulose blots. Myosin phosphorylation was expressed as the percentage of total MLC (15, 41).

**Calponin and caldesmon determination.** The relative calponin and caldesmon contents of ESO and LES were measured by immunostaining Western blots and normalized to either actin or dry tissue weight. Tissue slices were prepared from the circular smooth muscle layer of the ESO and LES, as described for single cell digestion. Each slice was carefully dropped into liquid nitrogen and stored at −70 °C until use. Tissue was homogenized under liquid nitrogen, dehydrated in acetone on ice, and dried to a powder. All tissue weights reported are tissue dry weights. Proteins were solubilized by incubation for 2 min at 100 °C in buffer containing 65.5 mM Tris base, 3% SDS, 20% glycerol, and 40 mM dithiothreitol. LES (15 μg) and ESO (12 μg) samples were processed by 5% SDS-PAGE using the buffer system of Porzio and Pearson (66) and electrophoretically transferred onto nitrocellulose. The nitrocellulose blots were immunostained using anti-rat uterus caldesmon and calponin polyclonal antisera, and autoradiographic and densitometric scans were performed. The linearity of the autoradiographic and densitometric scans was tested using a dilution series of purified chicken gizzard calponin on the same gel.

Because we did not know the relative staining intensity of calponin and caldesmon from chicken gizzard and from our LES and ESO samples, we could not calculate absolute amounts of calponin and caldesmon present in our tissues. Instead, we have reported the relative amounts of calponin and caldesmon normalized to either tissue dry weight or actin content. The actin content was determined by running a series of loadings on a 7.5% Porzio and Pearson (66) SDS-PAGE (from 2.5 to 25 μg of LES and ESO dry weight) along with several lanes of BSA (2 μg) to establish linearity of actin measurements. Gels containing the actin and BSA bands were stained for 2 h with a stain containing 0.1% Coomassie blue R-250, 25% isopropanol alcohol, and 10% acetic acid and destained overnight in destaining solution containing 10% acetic acid and 30% methanol. Stained bands were quantified by densitometric gel scanning, and the amount of actin per dry tissue weight of ESO and LES was calculated. Corrections were made for the difference in staining intensity of actin and BSA by Coomassie blue (actin/BSA , 0.78:1).

**RESULTS**

**MLCK-dependent LES contraction.** We (9, 33, 70) have previously shown, in smooth muscle cells isolated by enzymatic digestion from the circular layer of the LES, that ACh-induced contraction in the LES is mediated through a calmodulin-dependent pathway and contraction of ESO circular muscle is mediated through a PKC-dependent pathway. Because calmodulin is known to activate MLCK and ESO contraction is calmodulin independent, we investigated whether MLCK was involved in ESO contraction. We exposed LES and ESO circular smooth muscle cells to MLCK purified from chicken gizzard, in the presence of calcium and calmodulin. LES and ESO smooth muscle cells were permeabilized and equilibrated in the presence of 1.3 μM calcium and 1 nM calmodulin to facilitate activation of the kinase. Figure 1 shows that MLCK caused a concentration-dependent contraction of LES cells (ANOVA, P < 0.01). The half-maximal response, calculated by logit transformation, was seen at 0.2 nM. MLCK-induced contraction of ESO was significantly less than that of LES under the same experimental conditions (ANOVA, P < 0.01), and a statistically significant relationship between MLCK concentration and ESO contraction could not be established (ANOVA, P > 0.05).

To confirm that MLCK plays a greater role in contraction of LES than of ESO circular smooth muscle, we tested the effect of MLCK inhibitors on ACh-induced contraction of smooth muscle cells (Fig. 2). Muscle cells were contracted by a maximally effective con-
The MLCK inhibitors quercetin, L-thyroxine, and ML-7 concentration-dependently inhibited ACh-induced contraction of LES muscle (ANOVA, \( P < 0.01 \)) but had no effect on ESO muscle contraction.

PKC-dependent contraction of ESO and LES. These results suggest that ACh-induced ESO contraction, which is PKC dependent, may be mediated through a pathway not regulated by calmodulin and MLCK. To test the possibility that PKC-induced contraction may not be regulated by calmodulin and MLCK, LES and ESO smooth muscle cells were contracted by a maximally effective concentration of the PKC agonist DG, i.e., \( 10^{-7} \) M for ESO (70) and \( 10^{-6} \) M for LES muscle cells. Quercetin (10^{-5} M), L-thyroxine (10^{-6} M), and ML-7 (10^{-6} M) had no effect on contraction of ESO and LES muscle cells. Values are means ± SE of 3 animals (except \( n = 4 \) animals for ML-7), with 30 cells counted for each animal.

MLC phosphorylation in ACh-induced contraction of LES and ESO. Because ACh-induced contraction of ESO and DG-induced contraction of ESO and LES, which are PKC dependent, were not affected by MLCK inhibitors, we tested whether ACh-induced contraction of both ESO and LES was associated with MLC phosphorylation. MLC phosphorylation increased concentration dependently in response to ACh in both LES and ESO circular muscle (ANOVA, \( P < 0.01 \); Fig. 4). Phosphorylation of LES but not ESO circular muscle was significantly reduced in the presence of the calmodulin inhibitor CGS-9343B (ANOVA, \( P < 0.01 \); Fig. 4). To confirm that ACh-induced MLC phosphorylation is calmodulin dependent in LES but not in ESO muscle, we used the structurally unrelated calmodulin inhibitor \( N-(6\text{-amino}h\text{exyl})\text{-5-chloro-1-napthalenesulfonamide (W-7). Figure 5A shows that W-7 nearly abolished ACh-induced MLC phosphorylation in LES circular muscle (ANOVA, \( P < 0.01 \)). In addition, we examined the effect of the PKC inhibitor chelerythrine on LES MLC phosphorylation and found that PKC inhibition had no effect. Conversely, ESO phosphorylation was significantly reduced by chelerythrine (ANOVA, \( P < 0.01 \)) and unaffected by W-7 (Fig. 5B).

To test whether exogenous MLCK phosphorylates myosin, we measured phosphorylation in permeabilized smooth muscle cells isolated by enzymatic digestion from the circular layer of the LES and ESO. Muscle cells were contracted by a maximally effective concentration of ACh (10^{-10} M). Quercetin, L-thyroxine, and ML-7 concentration-dependently inhibited ACh-induced contraction of LES muscle (ANOVA, \( P < 0.01 \)) but had no effect on ESO muscle contraction. Values are means ± SE of 3 animals, with 30 cells counted for each animal.
smooth muscle cells in calcium-free medium, after adding calcium and calmodulin but not MLCK, after adding calcium, calmodulin, and MLCK, or after adding MLCK alone. The data were expressed as percent phosphorylation of myosin, calculated as phosphorylated MLC/(phosphorylated MLC + unphosphorylated MLC). Figure 6A shows that LES phosphorylation of myosin was low under all of the conditions examined except in the combined presence of calcium, calmodulin, and MLCK where phosphorylation increased from 6 ± 4% in calcium-free medium to 25 ± 5% (ANOVA, P < 0.05) in the presence of calcium, calmodulin, and MLCK. In ESO, myosin phosphorylation increased from 11 ± 8% in calcium-free medium to 18 ± 5% in the combined presence of calcium, calmodulin, and MLCK (Fig. 6B). Although this slight increase in phosphorylation was not statistically significant, it mirrors the slight contraction induced by MLCK in permeable ESO cells (Fig. 1).

Caldesmon, calponin, and regulation of PKC-induced contraction. These data suggest that MLCK-dependent MLC phosphorylation may play a lesser role in the ESO than in LES. In ESO, contraction and myosin phosphorylation may be preferentially mediated, directly or indirectly, by PKC activation.

Fig. 4. MLC phosphorylation in ESO and LES circular muscle in response to ACh. Nonphosphorylated and phosphorylated forms of MLC were separated by electrophoresis and localized with antibodies against MLC. The relative amounts of phosphorylated and nonphosphorylated MLC were quantitated by densitometry. Myosin phosphorylation was expressed as %total MLC. A: MLC phosphorylation increased concentration-dependently in response to ACh in both LES (ANOVA, P < 0.01) and ESO circular muscle (ANOVA, P < 0.01). Values are means ± SE of 3 animals, with 3 measurements for each animal. B: phosphorylation of LES but not of ESO circular muscle was reduced by the calmodulin inhibitor CGS-9343B (10⁻⁵ M) (ANOVA, P < 0.01). Values are means ± SE of 2 animals, with 3 measurements for each animal.

Fig. 5. ACh-induced MLC phosphorylation in LES (A) and ESO (B) in the presence of PKC inhibitor chelerythrine or calmodulin inhibitor W-7. MLC phosphorylation is presented as the %increase in phosphorylation in response to a maximally effective concentration of ACh (10⁻⁵ M) compared with unstimulated smooth muscle. A: W-7 (10⁻⁵ M) nearly abolished ACh-induced MLC phosphorylation in LES circular muscle (ANOVA, P < 0.01), but the PKC inhibitor chelerythrine (10⁻⁵ M) had no effect. B: ESO phosphorylation was significantly reduced by chelerythrine (ANOVA, P < 0.01) and unaffected by W-7. A and B: values are means ± SE of 5 animals.

Fig. 6. MLCK-induced MLC phosphorylation in LES (A) and ESO permeabilized smooth muscle cells (B). MLC phosphorylation was determined in permeabilized smooth muscle cells to control cytosolic calcium concentration and to add the impermeant calmodulin (CaM, 1 nM) and MLCK (10⁻⁸ M). Data are expressed as %phosphorylation of myosin calculated as phosphorylated MLC/(phosphorylated MLC + unphosphorylated MLC). Representative Western blots are shown for each experiment. A: LES phosphorylation of myosin was low under all of the conditions examined, except in the combined presence of calcium, calmodulin, and MLCK (Fig. 6A). Although this slight increase in phosphorylation was not statistically significant, it mirrors the slight contraction induced by MLCK in permeable ESO cells (Fig. 1).
Table 1. Caldesmon and calponin in circular muscle of ESO and LES

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<th>Caldesmon Density</th>
<th>Calponin Density</th>
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<tr>
<td></td>
<td>µg dry wt</td>
<td>µg actin</td>
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<tr>
<td>ESO</td>
<td>1.1 ± 0.1</td>
<td>12.7 ± 1</td>
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<tr>
<td>LES</td>
<td>0.7 ± 0.1</td>
<td>9 ± 0.7</td>
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<tr>
<td>Ratio</td>
<td>1.51</td>
<td>1.42</td>
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Values for esophagus (ESO) and lower esophageal sphincter (LES) are means ± SE of 5 measurements. The relative densities of calponin and caldesmon were measured by immunostaining Western blots and were normalized to either actin or tissue dry weight.

Calponin and caldesmon are regulatory proteins associated with actin filaments in smooth muscle and are thought to be either direct or indirect targets of PKC. Calponin and caldesmon bind to actin, inhibiting Mg²⁺-ATPase of phosphorylated smooth muscle myosin, thereby preventing cross-bridge cycling and smooth muscle contraction. It has been proposed (38, 59, 89, 90) that when calponin and caldesmon are phosphorylated by kinases such as PKC, actin binding and ATPase inhibition are abolished, restoring cross-bridge cycling and smooth muscle contraction. We examined the role of these regulatory proteins in contraction of LES and ESO.

Table 1 demonstrates that LES and ESO contain approximately the same amount of calponin, but ESO contains ~50% more caldesmon than LES. These values have been normalized to either actin or tissue dry weight.

To examine the role of caldesmon and calponin in contraction of LES and ESO, we exposed smooth muscle cells to caldesmon and calponin purified from chicken gizzard. Muscle cells were contracted by a maximally effective concentration of ACh (10⁻⁹ M). ACh-induced contraction of ESO cells was concentration-dependently inhibited by caldesmon and calponin (ANOVA, P < 0.01; Fig. 7). However, caldesmon and calponin did not concentration-dependently inhibit ACh-induced contraction of LES cells (ANOVA, P > 0.5; Fig. 7).

In ESO cells, the caldesmon peptide GS17C, which acts as a caldesmon antagonist (44), reversed caldesmon- but not calponin-induced inhibition of ACh (Fig. 8). GS17C is a 18-amino-acid peptide that contains the calmodulin- and actin-binding sequence of caldesmon.

Exposure of ESO cells to GS17C alone produced a concentration-dependent contraction (Fig. 9). The half-maximal response, calculated by logit transformation, was seen at 1.3 µM. Contraction induced by GS17C was significantly less in LES cells than in ESO cells.
(ANOVA, $P < 0.01$; Fig. 9). XGS17C, a peptide containing the same amino acid residues as GS17C, but with a randomized sequence, did not cause contraction (Fig. 9).

These data suggest a regulatory role for caldesmon in ESO contraction. Because ESO contraction in response to ACh was not affected by MLCK inhibitors, we tested whether MLCK inhibitors affected caldesmon-dependent contraction of ESO smooth muscle cells. Cells were exposed to a maximally effective concentration of GS17C ($10^{-5}$ M; Fig. 8). GS17C-induced contraction of ESO or LES smooth muscle cells was not inhibited by the MLCK inhibitors quercetin, L-thyroxine, and ML-7, suggesting that contraction induced through inhibition of caldesmon may not be regulated by MLCK (Fig. 10).

Because caldesmon and calponin can affect ACh-induced contraction of ESO, which is PKC dependent, but not of LES, which is calmodulin dependent, we tested the role of caldesmon and calponin in DG-induced contraction of LES and ESO (Fig. 11). LES and ESO were contracted by a maximally effective concentration of the PKC agonist DG, in the presence of caldesmon and calponin. Figure 11 shows that DG-induced contraction was inhibited by caldesmon and calponin in both LES (ANOVA, $P < 0.01$) and ESO (ANOVA, $P < 0.01$), suggesting that these proteins regulate PKC-induced contraction.

**DISCUSSION**

**Calmodulin and MLCK- and PKC-dependent contraction.** Phosphorylation of MLC by calcium and calmodulin-dependent MLCK is believed to be the primary determinant of smooth muscle contractility. However, a dissociation of the relationship between the intracellular calcium concentration and tension and myosin phosphorylation has been reported (57, 76). Therefore, other mechanisms may act in concert with or without the participation of MLC phosphorylation. Recently, much attention has been focused on the role of the thin-filament, actin-binding proteins calponin and caldesmon in smooth muscle contraction (35, 36, 65). In the present study, we demonstrate that ACh-induced contraction of LES smooth muscle cells depends on calcium and calmodulin-dependent MLCK activation. Contraction of ESO smooth muscle cells is PKC dependent and may be mediated by activation of calponin and caldesmon.

We (9) have previously shown that LES contraction in response to a maximally effective concentration of ACh is mediated by M3 muscarinic receptors linked to a calmodulin-dependent pathway and spontaneous tone or contraction in response to low ACh level depends on PKC. In contrast, ACh-induced contraction of ESO muscle depends on M2 muscarinic receptor-induced activation of a calcium-independent PKC-ε (72, 73). In the ESO, ACh-induced contraction is inhibited by the PKC inhibitors H-7, calphostin C, and chelerythrine (70). In addition, in ESO smooth muscle, ACh stimulates the translocation of the PKC-ε isozyme, but not of other isozymes, from the cytosol to the membrane. This pathway does not involve calmodulin, because ESO muscle cells contract relatively little in response to exogenous calmodulin under the same conditions that cause maximal contraction of LES cells and the calmodulin inhibitors CGS-9343B and W-7 do not inhibit ACh-induced contraction of ESO cells (70). In addition, permeabilized ESO cells contract in response to DAG even in calcium-free medium containing 2 mM EGTA (70). This finding excludes participation of the classical calmodulin-activated MLCK in the PKC-induced contractile process, because of the absolute requirement for calcium to activate calmodulin. Nevertheless, exogenous calmodulin and MLCK may still cause contraction, but this pathway is less efficient or sensitive in the ESO than in LES. The reduced calmodulin-MLCK sensitivity of ESO contraction is

![Figure 10](http://ajpgi.physiology.org/)

Fig. 10. Effect of MLCK inhibitors on GS17C-induced contraction of LES and ESO smooth muscle cells isolated by enzymatic digestion and permeabilized by brief exposure to saponin. Smooth muscle cells were exposed to GS17C ($10^{-5}$ M) alone (control) or in the presence of the MLCK inhibitors quercetin ($10^{-5}$ M), L-thyroxine ($10^{-5}$ M), or ML-7 ($10^{-4}$ M). GS17C-induced contraction of ESO smooth muscle was unaffected by MLCK inhibition, suggesting that contraction induced through inhibition of caldesmon may not be regulated by MLCK. Values are means ± SE of 4 animals, with 30 cells counted for each animal.

![Figure 11](http://ajpgi.physiology.org/)

Fig. 11. Effect of calponin and caldesmon on contraction of LES (A) and ESO smooth muscle cells (B) isolated by enzymatic digestion and permeabilized by brief exposure to saponin. Muscle cells were contracted by a maximally effective concentration of DG ($10^{-7}$ M for ESO and $10^{-6}$ M for LES). Caldesmon and calponin concentration dependently inhibited DG-induced contraction of both LES and ESO smooth muscle (ANOVA, $P < 0.01$). Values are means ± SE of 3 animals, with 30 cells counted for each animal.
consistent with this view (Fig. 1). In LES cells permeabilized by saponin, addition of MLCK in the presence of calcium and calmodulin caused concentration-dependent contraction, with a maximal response at 10^{-8} M MLCK. When ESO cells are exposed to MLCK, under the same conditions in which LES cells contract, relatively little contraction occurs. The calcium concentration used in this experiment was the threshold amount required in our system to cause calcium and calmodulin-dependent contraction. The calmodulin concentration used (1 nM) is the dissociation constant of calmodulin binding to MLCK (4). Although the mode of action of exogenous MLCK is not entirely clear, it is plausible that MLCK, in the presence of appropriate calcium and calmodulin concentrations, may phosphorylate MLC and cause contraction even if ACh-induced contraction of ESO cells depends on PKC activation and does not utilize this pathway. This view was confirmed in Fig. 6, A and B, in which MLCK-induced myosin phosphorylation was examined. The MLCK-induced phosphorylation data are in agreement with MLCK-induced contraction data. In the ESO, exogenously added MLCK, in the presence of sufficient calcium and calmodulin, causes lower levels of MLC phosphorylation than in the LES and causes only a slight contraction in ESO circular smooth muscle. In contrast, MLC phosphorylation and contraction in response to ACh are comparable in LES and ESO, as shown in Fig. 4.

Why MLCK fails to fully contract ESO muscle cells or phosphorylate ESO myosin is unclear. It is unlikely that the lack of effect of purified chicken gizzard MLCK on ESO cells may depend on a different MLCK structure in ESO. MLCK has been isolated from several smooth muscle tissues and characterized (43, 82). The amino acid sequences of MLCK from chicken gizzard (62), rabbit uterine (21), and bovine stomach smooth muscles (51) have been deduced from the corresponding cDNA sequences. These sequences are highly conserved even between different species (81). MLCK exhibits a high degree of substrate specificity (46), and there is no difference in substrate recognition properties of avian or mammalian MLCK (15). On the other hand, it is possible that some difference may exist between the MLC of ESO and LES, allowing recognition of LES but not of ESO MLC by chicken gizzard MLCK.

Our data suggest that contraction of ESO muscle depends less on MLCK than contraction of LES muscle. Several putative MLCK inhibitors, which inhibit the maximal LES contractile response to ACh, have no effect on ACh-induced contraction of ESO smooth muscle. Quercetin, a flavonoid, and ML-7 are two structurally different compounds that selectively and potently inhibit MLCK by binding hydrophobically at or near the ATP-binding site at the active center of the enzyme (32, 68). l-Thyroxine binds to the calmodulin-binding site of MLCK, inhibiting the binding of the calcium-calmodulin complex to the enzyme (29).

Different levels of phosphatase activity may be present in ESO and LES. If ESO muscle is heavily regulated by phosphatase activity, it may be less sensitive to activation of MLCK than to inactivation of phosphatase. Activation of PKC, and production of AA in response to ACh, may result in inactivation of MLC phosphatase (16), causing contraction. In permeabilized smooth muscle obtained from the rabbit femoral artery, PKC activation by phorbol esters and short-chain synthetic DAGs significantly increases force development and myosin phosphorylation of serine-19, the site phosphorylated by MLCK. In addition, the effect of phorbol ester is enhanced by the phosphatase inhibitor microcystin LR, suggesting that PKC activation increases MLC phosphorylation and force development through inhibition of MLC phosphatase (54). However, in our system, PKC activation by DG produced a contraction in both ESO and LES cells that was unaffected by MLCK inhibitors, suggesting that MLC phosphorylation induced by MLCK does not play a major role in PKC-mediated contraction. These data are in agreement with Horowitz et al. (36) who report that stimulation of ferret aortic smooth muscle cells with a constitutively active form of PKC-e results in contraction that is reversed by a selective PKC inhibitor but not by an MLCK inhibitor.

MLC is phosphorylated in calmodulin and MLCK and PKC-dependent contraction. Because MLCK activation is not directly responsible for PKC-dependent contraction, we examined whether ACh-induced contraction of ESO is associated with MLCK phosphorylation. Phosphorylation of the 20-kDa MLC during contraction of cat ESO and LES muscle was previously reported by Weisbrodt and Murphy (85), who found that phosphorylation increased in both LES and ESO during development of contraction. Phosphorylation reached a maximum before contraction, with maximum phosphorylation occurring at approximately two-thirds of the time required to achieve maximum contraction in both ESO and LES muscle (85). After reaching a peak, phosphorylation declined even though contraction persisted (85), and this behavior is consistent with data in other smooth muscles (6, 22, 30, 55, 80). Our data are in agreement with these findings. We find that contraction of ESO muscle peaks at 10 s after injection of ACh into the muscle chamber, whereas LES contraction peaks at 15 s. We measured myosin phosphorylation at 7 s for ESO and 10 s for LES, i.e., at two-thirds of the time required for contraction to reach its maximal value (85). At these times, we found that phosphorylation of the 20-kDa MLC increased concentration-dependently in both ESO and LES muscle. In addition, we report that LES, but not ESO, phosphorylation was antagonized by the calmodulin inhibitors CGS-9343B and W-7. The selective inhibitory effect of CGS-9343B shown in the current study correlates well with data previously reported in which CGS-9343B selectively inhibits the contractile response to a maximally effective concentration of ACh (10^{-8} M) in the LES (9) and not in the ESO (70). CGS-9343B selectively inhibits calmodulin by binding to the hydrophobic region of the calmodulin molecule; it does not inhibit PKC and only weakly affects PKA (61).
contrast, in ESO, phosphorylation was almost abolished by the PKC inhibitor chelerythrine and not by W-7.

MLC phosphorylation was measured under free-floating (auxotonic) conditions. We (9) previously found that under these conditions LES tissue produces inositol 1,4,5-trisphosphate (IP$_3$) in basal conditions and in response to ACh (73), suggesting that, although IP$_3$ levels may be higher when measured in conditions of isometric stretch, a measurable level is produced in auxotonic conditions. We measured MLC phosphorylation under isometric stretch, by tying muscle strips to a Plexiglas rod, and found that under these conditions phosphorylation was slightly higher than under free-floating conditions (data not shown). Percent changes in ACh-induced phosphorylation, however, were the same under either condition.

The phosphorylation studies show that ACh-induced MLC phosphorylation (similar to ACh-induced contraction) is the same in LES and ESO. When purified MLCK is used, however, MLCK-induced phosphorylation (and MLCK-induced contraction) is significantly greater in LES than ESO. These findings suggest that the classical MLCK may not be the major kinase responsible for phosphorylation of MLC (and contraction) in ESO and raise a question about the identity of the kinase responsible for phosphorylating ESO muscle MLC. Because ESO contraction and MLC phosphorylation (Fig. 5B) are PKC dependent and mediated through a calcium-independent PKC-ε isofrom (70), activation of PKC-ε may result in MLC phosphorylation through a process that may not involve an increase in activity of calcium and calmodulin-dependent MLCK. Although PKC may directly phosphorylate MLC, PKC phosphorylation sites of the 20-kDa MLC (serine-1, serine-2, threonine-3) are different from the MLCK phosphorylation sites (serine-18, serine-19) and inhibit rather than stimulate contraction (7, 37, 60, 77). Thus it is likely that PKC-induced muscle contraction may not result from direct PKC-induced phosphorylation of MLC (15), but that intermediate mechanisms may be activated in response to PKC (47–49). A chicken gizzard calcium-independent MLCK has been recently separated from MLCK by differential extraction from myofilaments and calmodulin affinity chromatography (84). This calcium-independent kinase associated with the myofilaments and distinct from MLCK has been shown to mediate contraction induced by the phosphatase inhibitor microcystin. Microcystin-induced contraction correlated with phosphorylation of MLC at serine-19 and threonine-18 (84). In ESO circular muscle, however, phosphatase inhibitors cause contraction by activating the calcium-independent PKC-ε (50). Thus it is probable that a different kinase may produce MLC phosphorylation in PKC-dependent contraction of ESO and LES circular muscle.

Calponin and caldesmon regulation of PKC-induced contraction. There is accumulating evidence for a secondary pathway of contraction that is mediated by PKC activation of calponin and caldesmon, two regulatory proteins associated with actin filaments in smooth muscle (35, 36, 65). Calponin and caldesmon bind to actin and inhibit the Mg$^{2+}$-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 was first described in 1981 (69) as a major protein component of chicken-gizzard smooth muscle that interacts with calmodulin in a calcium-dependent manner. Caldesmon interacts in a calcium-independent manner with actin, tropomyosin, and myosin (26, 39, 69). Caldesmon colocalizes in situ with actin, particularly with the contractile actin domain, consistent with a role in the regulation of contraction (20). The tissue content of caldesmon is variable, with tonic vascular smooth muscle containing less caldesmon (1 caldesmon: 205 actin monomers) than phasic smooth muscle (1 caldesmon: 22–28 actin monomers; Ref. 27). Our data are consistent with this finding: LES contains 50% less caldesmon than ESO smooth muscle that does not maintain tone.

Calponin is a 34-kDa, smooth muscle-specific protein that shares many properties with caldesmon. Shared properties are as follows: 1) calponin binds to actin and tropomyosin in a calcium-independent manner and to calmodulin in a calcium-dependent manner (78, 79); 2) calponin colocalizes with actin and tropomyosin in isolated smooth muscle cells (89), is found on thin filaments in situ, and is recovered in native thin-filament preparations (58, 83); 3) calponin inhibits myosin Mg$^{2+}$-ATPase in a reconstituted contractile system (90); and 4) the inhibitory effects of calponin are abolished by phosphorylation.

In the current study, we demonstrate a possible role of caldesmon and calponin in PKC-dependent contraction, as it occurs in ESO in response to ACh or DG (as either one results in activation of PKC; Refs. 13, 70, and 75) or in LES in response to DG. LES contraction in response to a maximally effective dose of ACh (10$^{-9}$ M) is calmodulin- and MLCK dependent and PKC independent (9) and is not affected by either caldesmon or calponin.

Addition of caldesmon to permeable smooth muscle cells concentration-dependently inhibits ACh-induced contraction. GS17C concentration-dependently induced contraction of ESO smooth muscle cells. GS17C is a peptide containing an 18-residue sequence of caldesmon (glycine-651 and serine-667) with an artificial cysteine residue added at the COOH terminus and contains both the calmodulin- and actin-binding sites of caldesmon. GS17C is a competitive antagonist of endogenous caldesmon that competes with caldesmon at the strong binding site for actin without having any effect, by itself, to inhibit myosin ATPase activity (44). GS17C has been shown to induce contraction of permeabilized ferret aortic cells with an EC$_{50}$ (0.92 μM) similar to our data (44). ACh-induced contraction of ESO cells may result from agonist-induced inhibition of endogenous caldesmon because addition of GS17C...
to cells precontracted with ACh does not result in additional contraction. In addition, GS17C reversed caldesmon-induced inhibition of ACh. Furthermore, GS17C-induced contraction, similar to PKC-induced contraction of ESO muscle, may be insensitive to activation of MLCK because it was not affected by MLCK inhibitors.

We (74) have previously demonstrated that in the ESO, ACh stimulates receptor-mediated activation of phospholipase A2, phospholipase D (71, 73), and phosphatidylinositol-specific phospholipase C (71, 73), producing AA and DAG. AA and DAG act synergistically and calcium-independently to activate PKC-ε (74). It is possible that PKC-ε activation in ESO smooth muscle may result, directly or indirectly, in the phosphorylation of caldesmon. When caldesmon is phosphorylated, actin binding and ATPase inhibition are abolished, restoring cross-bridge cycling and smooth muscle contraction.

Evidence (2, 3, 48) suggests that the mitogen-activated protein kinase (MAPK) may play a role in caldesmon regulation. Caldesmon is phosphorylated by MAPK in vitro and at the same sites as intact canine aortic strips treated with phorbol esters. These sites are near the COOH-terminal domain of caldesmon, which interacts with tropomyosin and actin. Caldesmon can reduce mean actin sliding velocity, and this inhibition is reversed by phosphorylation of caldesmon by p44 MAPK (23). PKC, Ras, Raf, MAPK kinase, and caldesmon have all been identified in aortic smooth muscle. Carbachol stimulation of airway smooth muscle increases caldesmon phosphorylation, and purified caldesmon is a substrate for activated murine extracellular signal-related kinase 2 (ERK2) MAPK (24). In gastrointestinal smooth muscle, a protein kinase cascade is activated by contractile agonists, which activates ERK MAP kinases, leading to phosphorylation of caldesmon (25). Both PKC-ε and MAPK translocate from the cytosol to the sarcolemma in response to α-adrenergic stimuli; PKC-ε remains associated with the sarcolemma whereas MAPK redistributes to the cytosol coincident with contraction (47, 48). It is possible that in the ESO, MAPK activation results in phosphorylation of caldesmon and contraction. This remains to be tested.

The role of calponin in contraction of ESO smooth muscle is less well defined. Calponin, similar to caldesmon, concentration-dependently inhibited Ach-induced contraction of ESO smooth muscle cells and PKC-dependent contraction of both LES and ESO. The physiological role of endogenous calponin was not examined in our investigation. However, the literature (35) supports a regulatory role of calponin in smooth muscle contraction. Treatment of aortic smooth muscle cells with a peptide corresponding to leucine-166 and glycine-194 of calponin produces a concentration-dependent contraction. This calponin peptide, which includes the actin-binding domain but excludes the actomyosin ATPase inhibitory region, presumably induces contraction by alleviating the inhibitory effect of calponin (35). Phosphorylation of both calponin and myosin increase in intact smooth muscle tissue strips when contracted by carbachol or the phosphatase inhibitor okadaic acid (14). Phenylephrine stimulation of single cells isolated from ferret portal vein activates a PKC-dependent pathway, resulting in a redistribution of calponin from the cytosol to the surface cortex. This agonist-induced redistribution of calponin was partially inhibited by the PKC inhibitor calphostin, overlapped in time with PKC translocation, and preceded contraction of these cells (63). In addition, a constitutively active form of PKC-ε has been shown (36) to phosphorylate both caldesmon and calponin. These studies suggest a possible physiological role of calponin in mediating agonist-induced PKC-dependent contraction.

We conclude that in LES and ESO muscle there are two distinct types of contractions, mediated by distinct regulatory mechanisms. A PKC-dependent pathway, perhaps regulated by caldesmon and calponin, is present in ESO and LES. A calmodulin and MLCK-dependent pathway is present only in LES muscle. MLCK phosphorylation occurs in both pathways.

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