Ca\(^{2+}\)-induced contraction of cat esophageal circular smooth muscle cells

W. CAO,\(^1\) Q. CHEN,\(^1\) U. D. SOHN,\(^2\) N. KIM,\(^3\) M. T. KIRBER,\(^1\) K. M. HARNETT,\(^1\) J. BEHAR,\(^1\) AND P. BIANCANI\(^1\)

Departments of Medicine, Rhode Island Hospital and Brown Medical School, Providence, Rhode Island 02903; \(^1\) Department of Pharmacology, College of Pharmacy, Chung Ang University, Seoul 156-756, and \(^3\) Department of Internal Medicine, Kangnam General Hospital, Public Corporation, Seoul 135-090, Korea

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Ca\(^{2+}\)-induced contraction of cat esophageal circular smooth muscle cells. Am J Physiol Cell Physiol 280: C980–C992, 2001.—ACh-induced contraction of esophageal circular muscle (ESO) depends on Ca\(^{2+}\) influx and activation of protein kinase C\(\varepsilon\) (PKC\(\varepsilon\)). PKC\(\varepsilon\), however, is known to be Ca\(^{2+}\)-independent. To determine where Ca\(^{2+}\) is needed in this PKC\(\varepsilon\)-mediated contractile pathway, we examined successive steps in Ca\(^{2+}\)-induced contraction of ESO muscle cells permeabilized by saponin. Ca\(^{2+}\) (0.2–1.0 \(\mu\)M) produced a concentration-dependent contraction that was antagonized by antibodies against PKC\(\varepsilon\) (but not by PKC\(\beta\) II or PKC\(\gamma\) antibodies), by a calmodulin inhibitor, by MLCK inhibitors, or by GDP\(\beta\)S. Addition of 1 \(\mu\)M Ca\(^{2+}\) to permeable cells caused myosin light chain (MLC) phosphorylation, which was inhibited by the PKC inhibitor chelerythrine, by D609 (phosphatidylcholine-specific phospholipase C inhibitor), and by propranolol (phosphatidic acid phosphohydrolase inhibitor). Ca\(^{2+}\)-induced contraction and diacylglycerol (DAG) production were reduced by D609 and by propranolol, alone or in combination. In addition, contraction was reduced by AACOCF\(_3\) (cytosolic phospholipase A\(_2\) inhibitor). These data suggest that Ca\(^{2+}\) may directly activate phospholipases, producing DAG and arachidonic acid (AA), and PKC\(\varepsilon\), which may indirectly cause phosphorylation of MLC. In addition, direct G protein activation by GTP\(\gamma\)S augmented Ca\(^{2+}\)-induced contraction and caused dose-dependent production of DAG, which was antagonized by D609 and propranolol. We conclude that agonist (ACh)-induced contraction may be mediated by activation of phospholipase through two distinct mechanisms (increased intracellular Ca\(^{2+}\) and G protein activation), producing DAG and AA, and activating PKC\(\varepsilon\)-dependent mechanisms to cause contraction.

calcium; smooth muscle; protein kinase C; phospholipase C; phospholipase D; myosin phosphorylation

MYOSIN LIGHT CHAIN KINASE (MLCK), in the presence of Ca\(^{2+}\) and calmodulin, phosphorylates the 20-kDa myosin light chain, causing contraction. The relationship between increased Ca\(^{2+}\) concentration, activation of the calmodulin-myosin light-chain kinase pathway, and contraction has been extensively examined. It has been proposed that MLCK plays an essential role in the activation process in the smooth muscle cell, so that activation of this enzyme is both necessary and sufficient for the initiation of contraction (31). Because the magnitude of changes in intracellular Ca\(^{2+}\) is not directly related to the force developed (27, 42, 52), an effort has been made to characterize the “Ca\(^{2+}\)-sensitivity” of the contractile process by proposing that Ca\(^{2+}\)-sensitivity may be modified by a number of factors, including activation of trimeric G proteins (19, 27, 35, 38, 42, 44, 52) and monomeric or “small” G proteins, and resulting in inhibition of phosphatases (19, 36, 40, 63) or in modulation of MLCK (2, 65, 67).

Although these factors affect the amplitude of contraction, a view of the contractile process as a MLCK-dependent relationship between Ca\(^{2+}\) and contraction excludes the possibility that contraction may occur through calmodulin-MLCK-independent pathways. Data from our laboratory (Table 1) suggest that calmodulin and MLCK play a role in ACh-induced lower esophageal sphincter (LES) contraction but not in contraction of esophageal circular muscle (ESO) (Table 1), which is mediated by activation of protein kinase C\(\varepsilon\) (PKC\(\varepsilon\)) (61). PKC\(\varepsilon\) is Ca\(^{2+}\)-independent, and diacylglycerol (DAG)-induced activation of permeable ESO cells can occur in Ca\(^{2+}\)-free medium (58). It is thus unlikely that this ESO contraction may be mediated by MLCK, which requires Ca\(^{2+}\)/calmodulin to be activated. In addition, ACh- or DAG-induced contraction of ESO cells is not affected by either calmodulin or MLCK inhibitors (Table 1), and permeable ESO cells do not contract well in response to purified calmodulin or MLCK at concentrations that cause pronounced contraction of LES cells (Sohn UD, Tang DC, Stull JT, Haeberle JR, Wang C-LA, Harnett KM, and Biancani P, unpublished observations).

To understand this calmodulin-MLCK-independent contraction, we investigated Ca\(^{2+}\)-induced contraction in ESO to identify the step in the contractile pathway

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that was dependent on the presence of Ca\(^{2+}\) to initiate contraction. We have previously shown that ACh-induced contraction of ESO depends on influx of Ca\(^{2+}\) but is mediated through a Ca\(^{2+}\)-insensitive PKCε-dependent pathway (7, 58, 61). In addition, we have shown that Ca\(^{2+}\) is required for production of the second messenger DAG. DAG, however, does not need Ca\(^{2+}\) for activation of the phospholipases responsible for production of second messengers. After the second messengers are produced, contraction may proceed through a pathway, which is Ca\(^{2+}\)-independent.

The data support this hypothesis: Ca\(^{2+}\) causes dose-dependent contraction of ESO cells through a PKCε-dependent pathway, resulting from Ca\(^{2+}\)-induced activation of phospholipases, production of second messengers, and phosphorylation of myosin light chains.

**METHODS**

**Tissue dissection and dispersion of smooth muscle cells.** Adult cats of either sex weighing 3–5 kg were euthanized, and esophageal smooth muscle squares from the circumferential muscle layer were prepared as previously described (7). The chest and abdomen were opened with a midline incision exposing the esophagus and stomach. The esophagus and stomach were removed together and pinned on a wax block at their in vivo dimensions and orientation. The esophagus and stomach were opened along the lesser curvature. After opening the esophagus and stomach and identifying the LES, we removed the mucosa and submucosal connective tissue by sharp dissection. The LES was excised, and a 3- to 5-mm-wide strip at the junction of the LES and esophagus was discarded to avoid overlap. The circular muscle layer from the esophagus was cut into 0.5-mm-thick slices with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA). The last slices containing the myenteric plexus, longitudinal muscle, and serosa were discarded, and slices were cut by hand into 2 × 2-mm tissue squares.

Tissue squares were digested in HEPES buffer, containing 0.1% collagenase type II to isolate smooth muscle cells, as previously described (7). The HEPES-buffered solution contained 112.5 mM NaCl, 5.5 mM KCl, 2 mM KH\(_2\)PO\(_4\), 10.8 mM glucose, 24 mM HEPES sodium salt, 1.87 mM CaCl\(_2\), 0.6 mM MgCl\(_2\), 0.3 mg/ml basal medium Eagle amino acid supplement, and 0.08 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 200-μm nylon mesh (Tetko, Elmsford, NY), rinsed in collagenase-free HEPES buffer to remove any trace of collagenase, and incubated in this solution at 31°C, gassed with 100% O\(_2\). The cells were allowed to dissociate freely for 10–20 min.

Cells were permeabilized, when necessary, to control intracellular Ca\(^{2+}\) concentration or to allow the use of agents such as antibodies, guanosine 5'-O-(3-thiotriphosphate); MLCK, myosin light chain kinase.
curves, cells were permeabilized in cytosolic medium containing 0 CaCl₂ and 1 mM EGTA. Cells were contracted by 30-s exposure to different Ca²⁺ concentrations calculated by the method of Fabiato and Fabiato (16). For generation of GTP·S concentration-response curves, cells were permeabilized in the indicated concentration of Ca²⁺, and then contracted by 30-s exposure to the indicated concentration of GTP·S.

When inhibitors (AACOCF₃, chelerythrine, CGS9334B, D609, ML-7, propranolol, quercetin) were used, the cells were incubated in appropriate concentrations of the inhibitors for 1 min before addition of agonist. Inhibitors were used at their maximally effective doses, as previously determined in pilot studies. When PKC antibodies or MLCK antibodies were used, permeabilized cells were incubated with the antibody at a 1:200 dilution for 60 min before addition of Ca²⁺. Thirty seconds after the agonist was added, the cells were fixed in acrolein at a final 1.0% concentration. A drop of the cell-containing medium was placed on a glass slide, 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, Secaucus, NJ) connected to a Macintosh computer (Apple, Cupertino, CA) with an image analysis software program (NIH Image 1.6; National Institutes of Health, Bethesda, MD; http://128.231.98.16/NIH-image/download.html). For each experiment, contraction was expressed as percent shortening of the average of 30 consecutive cells compared with the average of 30 untreated (control) cells. The average cell length of unstimulated cells was 70–75 µm. We have previously shown that permeabilization did not affect initial cell length or response to agonists (58).

Myosin phosphorylation. For measurement of myosin phosphorylation, permeabilized smooth muscle cells of the ESO were prepared as described above and preincubated in modified cytosolic buffer at 31°C for 20 min. Cells were then incubated with 1 mM Ca²⁺ for 10 s. The reaction was stopped by freezing the cells in a slurry containing acetone (90%), trichloroacetic acid (10%), 1 mM dithiothreitol, and dry ice.

Nonphosphorylated and phosphorylated forms of myosin light chain were separated by electrophoresis and localized with antibodies against myosin light chain. The relative amounts of phosphorylated and nonphosphorylated myosin light chain were quantitated by densitometry (13, 30). Briefly, protein was extracted in an 8 M urea buffer and processed for urea/glycerol-polyacrylamide gel electrophoresis as described in Ref. 48. Nonphosphorylated and phosphorylated forms of the light chain were separated following electrophoresis at 20°C and 30 mA for 4–6 h. Proteins were electrophoretically transferred from glycerol gels onto nitrocellulose paper. Myosin light chains were localized on nitrocellulose paper with antibodies against myosin light chain.

Relative amounts of phosphorylated and nonphosphorylated myosin light chain were quantitated from densitometry scans of the immunostained nitrocellulose blots. Myosin phosphorylation was expressed as percent of total myosin light chain (13, 30).

Cytosolic Ca²⁺ measurements. Freshly digested cells were placed on a shallow muscle chamber mounted on the stage of an inverted microscope (Carl Zeiss). The cells were allowed to settle to the bottom of the chamber. ACh was “spritzed” directly on the cells using a pressure ejection micropipette system.

Ca²⁺ measurements were obtained using a dual excitation wavelength imaging system (IonOptix Milton, MA). The Ca²⁺ concentrations were obtained from the ratios of fluorescence elicited by 340 nm excitation to 380 nm excitation using standard techniques (21). Fura 2 calibration was carried out using the potassium salt of fura 2 and solutions containing an excess of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) for determination of minimum ratio (R_{min}) and addition of saturating amounts of calcium for determination of maximum ratio (R_{max}). The dissociation constant (K_d) used was 224 nM. R_{max} and R_{min} were adjusted for in-cell conditions. R_{min} was measured in loaded cells permeabilized with ionomycin in the presence of excess BAPTA in the extracellular solution. The fractional decrease in R_{min} was measured to be 0.65 times the value measured in solution. R_{max} was also then corrected by this same factor. Background fluorescence was subtracted by defining a region outside of the cell and averaging the fluorescence in those pixels and subtracting that average from the fluorescence measured in the cell at each of the wavelengths. Additional autofluorescence in the cells was negligible. The ratiometric images were masked in the region outside the borders of the cell, since low photon counts can give unreliable ratios near the edges. An adaptive mask followed the borders of the cell after the Ca²⁺ changed and the cell contracted. A pseudoisoesthetic image (i.e., an image insensitive to Ca²⁺ changes) was formed in computer memory from a weighted sum of the images generated by 340 nm excitation and 380 nm excitation. This image was then thresholded, and values below a selected level were considered outside the cell and called 0. For each ratiometric image, the outline of the cell was determined and the generated mask was applied to the ratiometric image. This method allowed the simultaneous imaging of both the rapid changes in Ca²⁺ and cell length. Our algorithm was incorporated into the IonOptix software.

DAG measurements. Permeabilized smooth muscle cells of the ESO were prepared as described above and preincubated in modified cytosolic buffer at 31°C for 20 min. Cells were then incubated for 1 min in 0.5 ml modified cytosolic buffer alone, or containing the phospholipase inhibitors D609 (10⁻⁴ M), propranolol (10⁻⁴ M), or U-73122 (10⁻⁶ M). The cells were stimulated with calcium (1 mM) or GTP·S (10⁻⁶ M) for 40 s. The reaction was stopped with 3 ml chloroform-methanol (1:2 vol/vol). DAG was extracted by the addition of 1 ml of 1 M NaCl and 1 ml chloroform. The upper aqueous phase was discarded, and the lower organic phase was collected, evaporated under a stream of nitrogen, and frozen for DAG determination within 1 wk.

DAG measurements were performed using the Amersham DAG assay. Briefly, DAG was determined by incubating the samples with sn-1,2-diacylglycerol kinase in the presence of [³²P]ATP to form [³²P]phosphatidic acid. Lipids were then extracted with chloroform-methanol (1:2 vol/vol) and separated by high-performance thin-layer chromatography (HPTLC; Silica Gel 60; EM Science, Gibbstown, NJ) using a solvent system containing chloroform-methanol-acetic acid (5, 64). The presence of phosphatidic acid was determined by exposure of the HPTLC plate to X-ray film (X-OMat, XAR-2; Sigma, St. Louis, MO) overnight. Each HPTLC plate contained a control lane with standard DAG incubated with DAG kinase to document the precise location of phosphatidic acid. Quantitation of DAG was made by preparation of a standard curve with known concentrations of DAG. The spots containing [³²P]phosphatidic acid were cut from the HPTLC
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plate and placed into scintillation vials and assayed for radioactivity.

*Drugs and chemicals.* AACOCF₃ was purchased from Calbiochem; chelerythrine chloride from LC Services (Woburn, MA); collagenase type II and soybean trypsin inhibitor from Worthington Biochemicals (Freehold, NJ); fura 2 and BAPTA tetrashodmum salt from Molecular Probes (Eugene, OR); 1-(5-iodonaphthalene-1-sulfonyl)-H-hexahydro-1,4-diazepine hydrochloride (ML-7) from Seikagaku (Rockville, MD); MLCK and MLIC antibodies from Accurate Chemicals and Scientific (Westbury, NY); [³²P]ATP from New England Nuclear (Boston, MA); PKC antibodies (βII, γ, and ε) from GIBCO BRL (Gaithersburg, MD); SDS sample buffer from Bio-Rad (Hercules, CA); and U-73122 from Biomol (Plymouth Meeting, PA). ACh, ATP (disodium salt), antimycin A, BME amino acid supplement, creatine phosphate, creatine phosphokinase, EGTA, GDPβS, GTPγS (tetralithium salt), HEPES (sodium salt), propranolol, saponin, and other reagents were purchased from Sigma.

**Statistical analysis.** Data are expressed as means ± SE. Statistical differences between multiple groups were tested using ANOVA for repeated measures and checked for significance using Scheffe’s F-test.

**RESULTS**

Esophageal cells were permeabilized by saponin and exposed to increasing Ca²⁺ concentrations alone or in the presence of appropriate inhibitors. Figure 1A shows that Ca²⁺ caused a dose-dependent contraction (ANOVA, *P* < 0.001), with maximal contraction occurring at a 0.72 μM Ca²⁺. The contraction was almost completely inhibited by the PKC inhibitor chelerythrine (10⁻⁵ M; ANOVA, *P* < 0.001; Fig. 1A) and was not affected by the calmodulin inhibitor CGS9334B (Fig. 1B), by the putative MLCK inhibitors quercetin and ML-7, or by antibodies raised against MLCK (Fig. 1C), suggesting that, in ESO, Ca²⁺ activates a PKC-dependent, MLCK-independent contractile pathway.

To determine the specific PKC isozyme mediating Ca²⁺-induced contraction, we examined the effect of antibodies raised against the βII, γ, and ε PKC isozymes (61). Figure 2 shows that Ca²⁺-induced contraction of permeable ESO cells was not affected by PKCβII and PKCγ antibodies, but was significantly reduced by PKCε antibodies (ANOVA, *P* < 0.001), suggesting that, in ESO, Ca²⁺ activates a PKCε-dependent contractile pathway.

The PKCε-dependent mechanisms responsible for esophageal contraction have not been elucidated. Figure 3 shows that addition of 1 μM Ca²⁺ to permeable cells results in a significant increase in myosin light chain phosphorylation (ANOVA, *P* < 0.001), which was antagonized by PKC (ANOVA, *P* < 0.001) but not by calmodulin inhibitors. In addition, the increase in phosphorylation was antagonized by the phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin (10⁻⁵ M; ANOVA, *P* < 0.001), which was almost abolished by the protein kinase C (PKC) inhibitor chelerythrine (10⁻⁵ M; ANOVA, *P* < 0.001). The maximally effective concentration was 800–900 nM Ca²⁺. Ca²⁺-induced shortening was almost abolished by the protein kinase C (PKC) inhibitor chelerythrine (10⁻⁵ M; ANOVA, *P* < 0.001), which was almost abolished by the protein kinase C (PKC) inhibitor chelerythrine (10⁻⁵ M; ANOVA, *P* < 0.001). Values are means ± SE of 3 animals, with 30 cells counted for each data point.

**Fig. 1.** Ca²⁺-induced shortening of esophageal circular muscle (ESO) cells permeabilized by saponin. ESO cells were permeabilized by saponin to allow diffusion of extracellular Ca²⁺ into the cytoplasm. For generation of Ca²⁺ concentration-response curves, cells were permeabilized in cytosolic medium containing 0 CaCl₂ and 1 mM EGTA. Cells were contracted by 30-s exposure to different Ca²⁺ concentrations calculated by the method of Fabiato and Fabiato (16). A: the cells shortened in a dose-dependent manner in response to increasing Ca²⁺ concentrations (ANOVA, *P* < 0.001). The maximally effective concentration was 800–900 nM Ca²⁺. Ca²⁺-induced shortening was almost abolished by the protein kinase C (PKC) inhibitor chelerythrine (10⁻⁵ M; ANOVA, *P* < 0.001). B: at all Ca²⁺ concentrations shortening was not affected by the calmodulin inhibitor CGS9334B (10⁻⁵ M). C: Ca²⁺-induced contraction was not affected by the myosin light chain kinase (MLCK) inhibitors ML-7 (10⁻⁵ M), quercetin (10⁻⁵ M), or antibodies raised against MLCK (1:200). The “control” curve is the same as in A, as these experiments were performed in the same cell samples. Values are means ± SE of 3 animals, with 30 cells counted for each data point.
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Calcium and protein kinases. Figure 4 shows that activation of selected phospholipases, we used phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor D609 (ANOVA, \( P < 0.001 \)) and not by PKC\(_{\beta}II\) or PKC\(_{\gamma}\) antibodies. Values are means \( \pm \) SE of 4 animals, with 30 cells counted for each data point.

Figure 5 shows the effect of the phospholipase inhibitors D609 (10\(^{-4}\) M), propranolol (10\(^{-4}\) M), and U-73122 (10\(^{-6}\) M) on DAG production induced by 1 \( \mu \)M Ca\(^{2+}\) in permeable cells. In the absence of phospholipase inhibitors (control), DAG production was significantly increased by 61.53 \( \pm \) 13.7% when the intracellular Ca\(^{2+}\) concentration is increased from 0 to 1 \( \mu \)M (ANOVA, \( P < 0.05 \)). The Ca\(^{2+}\)-induced increase in DAG level was significantly lower in the presence of D609 (10\(^{-4}\) M) and propranolol (10\(^{-4}\) M) than in control samples (ANOVA, \( P < 0.05 \)). Propranolol and D609, in combination, completely abolished the Ca\(^{2+}\)-induced increase in DAG. U-73122 (10\(^{-6}\) M) did not significantly alter the Ca\(^{2+}\)-induced effect on DAG production. These data support the hypothesis that elevations in cytosolic Ca\(^{2+}\) are capable of activating PLD and PC-PLC, resulting in production of DAG. Values are means \( \pm \) SE of 4 animals.
lipase inhibitors (control), DAG production was significantly increased (61.53 ± 13.7%) when the intracellular Ca²⁺ concentration was increased from 0 to 1 μM (ANOVA, *P < 0.05). The Ca²⁺-induced increase in DAG level was reduced by D609 and propranolol (ANOVA, *P < 0.05). Propranolol and D609, in combination, completely abolished the Ca²⁺-induced increase in DAG. U-73122 did not significantly alter Ca²⁺-induced DAG production.

We have previously shown that ACh-induced esophageal contraction is mediated by activation of PC-PLC, PLD, and of a 100-kDa cytosolic phospholipase A₂ (cPLA₂). cPLA₂ produces arachidonic acid (AA) which potentiates DAG-induced activation of PKC (58). We therefore tested whether cPLA₂ may also participate in Ca²⁺-induced contraction of ESO cells. We found that the cPLA₂ inhibitor AACOCF₃ significantly reduced Ca²⁺-induced contraction by 79%, had no effect on Ca²⁺-induced DAG production, and of a 100-kDa cytosolic phospholipase A₂ (cPLA₂). The cPLA₂ inhibitor AACOCF₃ (10⁻⁴ M), which is known to activate muscarinic receptors linked to heterotrimeric G proteins, values are means ± SE of 3 animals, with 30 cells counted for each data point. Values are means ± SE of 3 animals, with 30 cells counted for each data point. These data suggest that G protein activation results in activation of phospholipases.

Fig. 7. Ca²⁺-induced shortening of permeable cells does not depend on activation of G proteins. Left: the nonhydrolyzable GDP analog guanosine 5′-O-(2-thiodiphosphate) (GDPβS) dose dependently reduced cell shortening (ANOVA, *P < 0.001), in response to a maximally effective dose of ACh (10⁻⁶ M), which is known to activate muscarinic receptors linked to heterotrimeric G proteins. Values are means ± SE of 3 animals, with 30 cells counted for each data point. Right: a 10⁻⁴ M concentration of GDPβS, which reduced ACh-induced contraction by 79%, had no effect on Ca²⁺-induced contraction. Values are means ± SE of 3 animals, with 30 cells counted for each data point.

Taken together, these data suggest that an increase in cytosolic Ca²⁺ may directly activate PC-PLC, PLD, and PLA₂, resulting in production of DAG and AA and activation of a PKC-dependent pathway, similar to the contractile pathway activated by ACh.

To test whether Ca²⁺-induced activation of phospholipases involves the activation of G proteins, we used the nonhydrolyzable GDP analog GDPβS. The effectiveness of GDPβS was tested against ACh, which is known to activate muscarinic receptors linked to trimeric G proteins. Figure 7 shows that contraction in response to a maximally effective dose of ACh was dose dependently reduced by GDPβS (ANOVA, *P < 0.001). GDPβS at a 10⁻⁴ M concentration reduced ACh-induced contraction by 79% and at a 10⁻³ M concentration by 90%. A 10⁻⁴ M concentration of GDPβS, however, had no effect on Ca²⁺-induced contraction (Fig. 7), supporting the view that Ca²⁺-induced contraction of ESO cells may result from directly induced activation of phospholipases, bypassing G proteins.

However, ACh binding to muscarinic receptors in ESO (60) causes not only elevation of cytosolic Ca²⁺ (56) but also activation of G proteins. We therefore examined whether the nonhydrolyzable GTP analog GTPγS and Ca²⁺ could interact in contraction of permeable ESO cells. Figure 8A compares contraction of permeable ESO cells in response to increasing concentrations of Ca²⁺ alone and in the presence of three concentrations of GTPγS (10⁻⁸, 10⁻⁷, and 10⁻⁶ M). When cytosolic Ca²⁺ levels reach 100 nM and higher, GTPγS augments Ca²⁺-induced contraction in a dose-dependent manner (ANOVA, *P < 0.0010). Figure 8B shows that propranolol and D609, in combination, almost completely abolish GTPγS-induced contraction, suggesting that G protein activation results in activation of phospholipases.

Fig. 6. Ca²⁺-induced contraction depends on activation of cytosolic phospholipase A₂ (cPLA₂). The cPLA₂ inhibitor AACOCF₃ (10⁻³ M) significantly reduced Ca²⁺-induced contraction (ANOVA, *P < 0.001), suggesting that cPLA₂ contributes to Ca²⁺-induced contraction of ESO cells. Values are means ± SE of 4 animals, with 30 cells counted for each data point.
nation with $10^{-6}$ M GTP$_S$, by 490 nM Ca$^{2+}$ with $10^{-7}$ M GTP$_S$, by 600 nM Ca$^{2+}$ with $10^{-8}$ M GTP$_S$, or by 700 nM Ca$^{2+}$ alone. To estimate which combination of cytosolic Ca$^{2+}$ and G protein activation occurs when ESO cells contract in response to the endogenous neurotransmitter ACh, we examined the Ca$^{2+}$ concentration after ACh stimulation.

Ca$^{2+}$ measurements were obtained with a dual wavelength Ca$^{2+}$ imaging system. The images at the top of Fig. 9 show sequential shortening and Ca$^{2+}$ levels in a single ESO cell at different times after exposure to ACh. The numbers below each image represent the time elapsed. Application of ACh by pressure ejection micropipette began at ~1 s and lasted 5 s. Figure 9 shows that the first detectable Ca$^{2+}$ rise occurs ~1.5 s after the beginning of application of ACh. Ca$^{2+}$ levels do not increase uniformly across the cell, but rather an area of elevated Ca$^{2+}$ sweeps from the bottom to the top of the cell in ~1 s, presumably as the applied ACh engulfs the cell from the bottom to the top. Visible shortening of the cell is maximal at 15–30 s and occurs after the peak of the Ca$^{2+}$ signal is past, when cytosolic free Ca$^{2+}$ has returned to near resting levels. A comparison of the pseudocolors observed in the cell with the scale on the right of the figure suggests that the highest peak of Ca$^{2+}$ concentration that sweeps this cell may be between 500 and 600 nM. This observation is confirmed by the graph in the lower panel. The graph represents the average Ca$^{2+}$ concentration as a function of time in a small window in the cell. A small window may be reasonably representative of the maximum Ca$^{2+}$ levels reached in the cell, as the same maximum level seems to occur throughout the cell, albeit at slightly different times. For the muscle cell shown in Fig. 9, the graph shows a peak 600 nM Ca$^{2+}$ concentration occurring at 3–4 s after application of ACh. When Ca$^{2+}$ was measured in 20 cells under identical conditions, the peak was 616 ± 50 nM (means ± SE). The Ca$^{2+}$ concentration decreases after the peak and returns to approximately prestimulation values at 29 s, the time of maximum shortening.

**DISCUSSION**

The data presented show that in ESO smooth muscle Ca$^{2+}$ directly activates phospholipases, causing production of second messengers and activation of a PKC-dependent contractile pathway. G protein activation by GTP$_S$ (or by muscarinic receptors linked to trimeric G proteins) activates the same phospholipases and contractile pathway. These findings may help in understanding a possible mechanism responsible for “modulation of Ca$^{2+}$ sensitivity” by GTP$_S$.

Ca$^{2+}$ is thought to be a universal messenger of intracellular signaling for a wide variety of cell processes. In smooth muscle an increase in cytoplasmic Ca$^{2+}$ leads to phosphorylation of the 20-kDa myosin light chains (62). However, smooth muscle cells contain numerous proteins that are capable of binding Ca$^{2+}$, either to buffer changes in ionized Ca$^{2+}$ or to elicit a cellular response (57), and the intermediate processes

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**Fig. 8.** Potentiation of Ca$^{2+}$-induced contraction by guanosine 5’-O-(3-thiotriphosphate) (GTP$_S$). A: contraction of permeable ESO cells in response to increasing concentrations of Ca$^{2+}$ alone (circles) is dose dependently increased by increasing concentrations of GTP$_S$ (10$^{-6}$, 10$^{-7}$, and 10$^{-6}$ M). In Ca$^{2+}$-free cytosolic medium GTP$_S$ caused no contraction, but, at 100 nM and higher cytosolic Ca$^{2+}$ levels, GTP$_S$ augmented Ca$^{2+}$-induced contraction in a dose-dependent manner. Values are means ± SE of 3 animals, with 30 cells counted for each data point. B: in the presence of 0.36 μM Ca$^{2+}$, contraction of ESO cells in response to increasing concentrations of GTP$_S$ is abolished by propranolol (10$^{-6}$ M) and D609 (10$^{-5}$ M), in combination, suggesting that activation of G proteins may lead to PLC-PLD stimulation. Values are means ± SE of 3 animals, with 30 cells counted for each data point. C: effect of GTP$_S$ on DAG production. Values are the percent increase in DAG levels in the presence of GTP$_S$ (10$^{-6}$ M). The intracellular Ca$^{2+}$ concentration was 180 nM. In the absence of phospholipase inhibitors (control), DAG production increased 38.94 ± 5.2% in the presence of GTP$_S$. The GTP$_S$-induced increase in DAG level was significantly lower in the presence of D609 (10$^{-4}$ M) and propranolol (10$^{-4}$ M) than in control samples (ANOVA, $P < 0.0010$). Propranolol and D609, in combination, completely abolished the GTP$_S$-induced increase in DAG. U-73122 (10$^{-5}$ M) did not alter the GTP$_S$-induced effect on DAG production (ANOVA, $P = 0.14$). These data support the hypothesis that elevations in GTP$_S$ are capable of activating PLD and PLC, resulting in production of DAG. Values are means ± SE of 4 animals.
initiated by Ca$^{2+}$ and leading to phosphorylation of the 20-kDa myosin light chains are not well understood.

Several investigators have shown that the magnitude of changes in intracellular Ca$^{2+}$ is not directly related to the force developed (27, 42, 52), and an effort has been made to characterize the Ca$^{2+}$ sensitivity of the contractile process by examining other possible modulators of Ca$^{2+}$ sensitivity, such as GTP$_S$ (17, 18, 35, 38, 44), phosphatases (19, 36, 37, 40), or modulation of MLCK (2, 64, 65).

A “Ca$^{2+}$-centric” view of the contractile process may not be helpful in understanding the sequence of intracellular events occurring between Ca$^{2+}$ influx, or release, and myosin phosphorylation. Often the sequence of events leading to contraction is complex and not amenable to explanation in terms of Ca$^{2+}$ sensitivity. This point is well illustrated by reports of Ca$^{2+}$-independent contraction in some vascular and esophageal muscles (28, 58, 67). Definition of the sequence of events and determination of the precise site of action of Ca$^{2+}$ in the sequence leading to contraction are needed.

We have previously shown that different intracellular processes mediate contraction in different muscles (59, 60) or even in the same muscle under different conditions (6). For instance, in the LES, contraction in response to a maximally effective dose of ACh is mediated by inositol trisphosphate (IP$_3$)-induced release of Ca$^{2+}$ from intracellular stores and activation of a calmodulin-MLCK-dependent pathway, but spontaneous tone or contraction in response to a low level of ACh depends on PKC and not on calmodulin (6, 26).

In contrast, contraction of ESO muscle in response to ACh depends on activation of phospholipases and production of DAG and AA, without any measurable production of IP$_3$ (58, 60). Once DAG and AA are produced, they interact to activate a Ca$^{2+}$-independent PKCε (58, 61), and in permeable cells DAG-induced contraction can occur even in Ca$^{2+}$-free solution. This pathway does not involve calmodulin or MLCK, which
require Ca\textsuperscript{2+} to be activated. In addition, ESO muscle cells do not contract in response to exogenous calmodulin or MLCK under the same conditions that cause maximal contraction of LES cells, and the calmodulin inhibitor CGS9343B or the MLCK antibody and inhibitors (quercetin or ML-7) do not inhibit ACh-induced contraction of ESO cells (Table 1) (Sohn UD, Tang DC, Stull JT, Haebelke JR, Wang C-LA, Harnett KM, and Biancani P, unpublished observations).

Ca\textsuperscript{2+} requirements exist even when contraction is mediated by the Ca\textsuperscript{2+}-independent PKCe, since contraction of ESO cells is almost abolished by prolonged exposure to Ca\textsuperscript{2+} channel inhibitors or to Ca\textsuperscript{2+} chelators such as EGTA (7, 58). In the present study we examined the mechanism mediating Ca\textsuperscript{2+}-induced contraction of ESO cells, isolated by enzymatic digestion and permeabilized by brief exposure to saponin, to allow control of cytosolic Ca\textsuperscript{2+} levels. We found that permeable esophageal cells contract in a dose-dependent manner as cytosolic Ca\textsuperscript{2+} concentration increases from 200 nM to micromolar. These data suggest that diffusion of Ca\textsuperscript{2+} into the cytoplasm is sufficient to produce contraction of esophageal muscle. In addition, similar to ACh-induced contraction in ESO (7, 58, 61), Ca\textsuperscript{2+}-induced contraction is mediated through a PKC-dependent pathway as it is almost abolished by chelerythrine (Table 1). Chelerythrine interacts with the catalytic domain of PKC and is a potent PKC inhibitor with a half-maximal inhibition occurring at 0.66 \muM (23). Ca\textsuperscript{2+}-induced contraction was not affected by the calmodulin inhibitor (25, 46, 49) CGS9343B, by two putative MLCK inhibitors, or by antibodies raised against MLCK (Fig. 1). Quercetin, a flavonoid, and ML-7 are two structurally different compounds that inhibit MLCK by binding hydrophobically at or near the ATP-binding site at the active center of the enzyme (22, 24, 54). We have previously shown that both ML-7 and quercetin cause dose-dependent inhibition of LES but not of ESO muscle cells in response to a maximally effective dose of ACh (Table 1), suggesting that both ML-7 and quercetin are selective enough to cause inhibition of LES but not of ESO. These data are consistent with the view that Ca\textsuperscript{2+}-induced contraction of ESO may be calmodulin- and MLCK-independent, and mediated through a PKC-dependent pathway. This hypothesis is also consistent with the finding that DAG-induced contraction of ESO-permeable cells is not affected by incubation in Ca\textsuperscript{2+}-free medium (58), as the presence of Ca\textsuperscript{2+} at relatively high concentration is needed to activate calmodulin and MLCK (6).

We have previously shown that the \beta II, \gamma, and \epsilon PKC isozymes are present in ESO circular muscle (61) and that only PKCe translocates from the cytosol to the membrane in response to ACh (61). In the present investigation we identified the isozyme activated by cytosolic Ca\textsuperscript{2+} elevation, by examining the effect of isozyme-selective PKC antibodies on Ca\textsuperscript{2+}-induced contraction. Figure 2 shows that contraction of permeabilized ESO cells was inhibited by antibodies raised against PKCe and not by antibodies raised against the \beta II or \gamma PKC isozymes (ANOVA, \(P < 0.01\)). Inhibition of Ca\textsuperscript{2+}-induced contraction by the PKCe antibody was concentration dependent and reversed by addition of the PKCe-specific antibody-binding peptide (Table 1). Thus, as in the case of ACh-induced contraction of ESO cells, Ca\textsuperscript{2+}-induced contraction results in activation of a PKCe-dependent contractile pathway. PKC is a family of homologous serine and threonine protein kinases that can be divided into three groups based on their Ca\textsuperscript{2+} and phospholipid requirements for activation: the classical or conventional PKC isozymes (\(\alpha, \beta I, \beta II, \gamma\)) are Ca\textsuperscript{2+} and phospholipid dependent; the new PKC isozymes (\(\delta, \epsilon, \eta, \theta, \mu\)) are Ca\textsuperscript{2+} independent and phospholipid dependent; and the atypical PKC isozymes (\(\zeta, \lambda\)) are Ca\textsuperscript{2+} and phospholipid independent (45). The new PKC isozymes (i.e., PKCe) lack the region that has been implicated in the regulation of PKC by Ca\textsuperscript{2+} (5, 29, 47). It is thus unlikely that Ca\textsuperscript{2+} may directly activate PKCe.

We have shown that ACh-induced contraction of ESO, which is PKCe dependent, is associated with myosin light chain phosphorylation (Sohn UD, Tang DC, Stull JT, Haeblerke JR, Wang C-LA, Harnett KM, and Biancani P, unpublished observations), which was inhibited by PKC inhibitors and not by calmodulin inhibitors. We therefore examined whether an increase in cytosolic Ca\textsuperscript{2+} may also result in PKC-dependent phosphorylation of myosin light chains. Figure 3 shows that Ca\textsuperscript{2+}-induced myosin light chain phosphorylation was antagonized by PKC but not by calmodulin inhibitors and by the PC-PLC inhibitor D609 and by the PLD pathway inhibitor propranolol. These data confirm that, like Ca\textsuperscript{2+}-induced contraction, Ca\textsuperscript{2+}-induced phosphorylation of myosin light chain depends on phospholipase-mediated production of DAG and activation of PKC, and not on activation of a calmodulin-dependent pathway.

Similarly, Ca\textsuperscript{2+}-induced contraction, like Ca\textsuperscript{2+}-induced myosin phosphorylation, was significantly reduced by the phospholipase inhibitors D609 and propranolol, when used separately, and was abolished by D609 and propranolol, when used in combination. We have previously shown that ACh-induced contraction of esophageal muscle is not associated with hydrolysis of phosphatidylinositol 4,5-bisphosphate and production of IP\textsubscript{3} (50). Phosphatidylcholine hydrolysis by PC-PLC and by PLD has been shown to be an alternative source of DAG (8, 15, 39, 66). PC-PLC produces DAG and phosphocholine, while PLD produces choline and phosphatidic acid, which is metabolized to DAG by phosphatidic acid phosphohydrolase (8, 14). D609 blocks PC-PLC activity derived from Bacillus cereus, without affecting PLA\textsubscript{2}, PLD, and PI-PLC activity (55). A high concentration (0.1–1 mM) of propranolol has been shown to reduce DAG production by inhibition of phosphatidic acid phosphohydrolase without affecting PLD activity or PI-PLC activity (9, 50, 51). Ca\textsuperscript{2+}-induced contraction of ESO muscle cells and production of DAG were antagonized by D609 and by propranolol at concentrations that had no effect on contraction...
of LES muscle (59, 60). The lack of effect of propranolol on contraction of LES muscle cells implies that, at the concentration used, propranolol is not acting nonselectively as a local anesthetic. This finding is consistent with the view that esophageal contraction may be mediated by activation of PC-PLC and PLD. The fact that inhibition by D609 and propranolol is additive, and results in complete abolition of esophageal contraction, suggests that this is the main signaling pathway responsible for contraction of esophageal circular muscle.

In addition, Ca$^{2+}$-induced production of DAG in response to 1 μM Ca$^{2+}$, similarly to cell contraction, was reduced by D609 and propranolol, when used alone, and abolished when the inhibitors were present in combination. U-73122, a selective inhibitor of PI-PLC, had no effect. These data confirm that, in the ESO, elevations in cytosolic Ca$^{2+}$ are capable of activating PLD and PC-PLC selectively.

Taken together, these data show that DAG formation, myosin light chain phosphorylation, and contraction all depend on Ca$^{2+}$-induced activation of PC-PLC and PLD, strongly supporting phospholipases as the site of action for Ca$^{2+}$ to activate a PKCε-dependent contractile pathway. In addition, activation of PKCε results in phosphorylation of MLCK, which is not calmodulin dependent. It is unlikely that PKCε directly phosphorylates MLCK. Other kinases, such as extracellularly regulated kinase (ERK) 1 and ERK2 (12), and other regulatory proteins, such as caldesmon and/or calponin (1, 3, 32, 33, 41, 43), are likely to be involved in PKC-mediated contraction.

A high-molecular-mass (85–110 kDa) cPLA$_2$ participates in contraction of esophageal but not of LES muscle by producing AA and potentiating DAG-induced activation of PKC (58). We therefore examined the role of cPLA$_2$ in Ca$^{2+}$-induced contraction of ESO circular muscle. AAOCCF$_3$, an analog of AA in which the COOH group is replaced with trifluoromethyl ketone (63), has been shown to selectively inhibit cPLA$_2$ in platelets (4, 20, 53) and mesangial cells (20). AAOCCF$_3$ significantly reduced Ca$^{2+}$-induced contraction of ESO circular muscle cells (Fig. 6). Inhibition of Ca$^{2+}$-induced contraction by AAOCCF$_3$ (10$^{-5}$ M) varies between 20% (at high Ca$^{2+}$) and 40% at (at 360 nM Ca$^{2+}$), is statistically significant and slightly lower than previously reported in response to ACh (34). It should be noted, however, that ACh causes both Ca$^{2+}$ influx (which can directly activate phospholipases) and G protein activation (which can independently activate the same phospholipases). When cPLA$_2$ is activated only by Ca$^{2+}$, it is reasonable to expect that its contribution may be less than when activated by ACh, as the effect arising from G protein activation is absent.

These data suggest that, in ESO circular smooth muscle, Ca$^{2+}$ functions by activating phospholipases, resulting in production of DAG and AA. Thus the signal transduction pathway activated by Ca$^{2+}$ elevation is the same as the one activated by ACh (58–60).

To determine whether Ca$^{2+}$-induced activation of phospholipases was direct or mediated by activation of G proteins, we used the GDP analog GDPβS to inhibit GTP binding to G proteins, and G protein activation. ACh is thought to be the endogenous neurotransmitter mediating contraction of esophageal muscle in physiological conditions, e.g., in response to swallowing. ACh-induced contraction of ESO is mediated by M2 muscarinic receptors linked to Gi3-type G proteins (60) and is inhibited by GDPβS. Figure 7 shows that GDPβS dose dependently reduced ACh-induced contraction but had no effect on Ca$^{2+}$-induced contraction of ESO cells. These data suggest that Ca$^{2+}$ may induce contraction by direct activation of phospholipases, without activation of G proteins.

Because ACh causes both elevation of cytosolic Ca$^{2+}$ and activation of G proteins, we examined the interaction of G protein activation by GTPγS and Ca$^{2+}$ in contraction of ESO. Figure 8A compares contraction of permeable ESO cells in response to increasing concentrations of Ca$^{2+}$ alone and in the presence of GTPγS. In the absence of Ca$^{2+}$, ESO cells do not contract in response to GTPγS (58). When cytosolic Ca$^{2+}$ levels reach 100 nM and higher, GTPγS augments Ca$^{2+}$-induced contraction in a dose-dependent manner (ANOVA, P < 0.0010). The additional contraction induced by GTPγS depends on G protein-induced activation of phospholipases, because propranolol and D609, in combination, almost completely abolish GTPγS-induced contraction (Fig. 8B). Similarly, these phospholipase inhibitors (but not U-73122) reduced GTPγS-induced production of DAG. The GTPγS-induced increase in DAG level was almost abolished by D609, by propranolol, and completely abolished by propranolol and D609 in combination. The PI-PLC inhibitor U-73122 did not alter DAG production.

Several investigators have observed that GTPγS increases Ca$^{2+}$ sensitivity of the contractile process (17, 18, 36–38, 44). Our data suggest that the G protein-independent contraction induced by Ca$^{2+}$ and the G protein-dependent contraction induced by GTPγS are both mediated by the same intracellular pathway, i.e., by inducing phospholipase activation and production of second messengers.

Figure 8A demonstrates that the same level of contraction may result from different combinations of Ca$^{2+}$ and GTPγS. At any Ca$^{2+}$ concentration, the contraction may be augmented by GTPγS (i.e., by G protein activation) in a dose-dependent manner. To estimate which combination of cytosolic Ca$^{2+}$ and G protein activation occurs when ESO cells contract in response to ACh, we measured cytosolic Ca$^{2+}$ levels in the ESO after ACh.

Ca$^{2+}$ measurements were obtained with a dual-wavelength Ca$^{2+}$ imaging system. The images in Fig. 9 show sequential shortening and Ca$^{2+}$ levels in a single ESO cell at different times after exposure to ACh. Application of ACh by pressure ejection micropipette began at ~1 s and lasted 5 s. Ca$^{2+}$ levels did not increase uniformly across the cell, but rather an area of
activated, contraction can occur in the absence of Ca\(^{2+}\) decreasing and was maximal at 15–30 s at a time when cytosolic free Ca\(^{2+}\) had returned to near resting levels. Contraction of intact cells in response to application of ACh by pressure ejection may not be directly comparable to contraction of permeable cells exposed to a fixed Ca\(^{2+}\) concentration, and the calculated Ca\(^{2+}\) concentrations used for permeable cells may not be directly comparable to the fura 2-measured concentrations. Nevertheless, analysis of the cell images shows a 26\% shortening, which was comparable to the shortening in permeable cells in response to Ca\(^{2+}\) or ACh, and in intact esophageal cells, in response to a variety of agonists. ACh-induced Ca\(^{2+}\) elevation was below the 700–800 nM levels required to produce maximal contraction in isolated esophageal cells, as shown in Figs. 1, 4, 6, and 8. Thus it is possible that ACh-induced contraction of ESO cells may result from interaction of Ca\(^{2+}\) and G proteins and that the relatively low Ca\(^{2+}\) concentration measured in response to ACh may be amplified by concurrent ACh-induced activation of G proteins.

We conclude that Ca\(^{2+}\) and G protein activation in ESO cells results in activation of the same phospholipases, producing the second messengers required to activate the Ca\(^{2+}\)-insensitive PKC\(\varepsilon\). Once PKC\(\varepsilon\) is activated, contraction can occur in the absence of Ca\(^{2+}\). Phospholipase activation by Ca\(^{2+}\) and G proteins may account in part for previously reported Ca\(^{2+}\) sensitization by GTP\(\gamma\)S. Under physiological conditions ACh-induced contraction may use a combination of both mechanisms to activate a contractile pathway.

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