Sustained muscle contraction induced by agonists, growth factors, and Ca^{2+} mediated by distinct PKC isozymes

K. S. Murthy, J. R. Grider, J. F. Kuemmerle, and G. M. Makhlouf. Sustained muscle contraction induced by agonists, growth factors, and Ca^{2+} mediated by distinct PKC isozymes. Am J Physiol Gastrointest Liver Physiol 279: G201–G210, 2000.—The role of protein kinase C (PKC) in sustained muscle contraction was examined in intestinal circular and longitudinal muscle cells. Initial contraction induced by agonists (CCK-8 and neuromedin C) was abolished by inhibitors of Ca^{2+} mobilization (neomycin and dimethyleicosadienoic acid), 2) calmidazolium, and 3) myosin light chain (MLC) kinase (MLCK) inhibitor KT-5926. In contrast, sustained contraction was not affected by these inhibitors but was abolished by 1) the PKC inhibitors chelerythrine and calphostin C, 2) PKC-ε antibody, and 3) a pseudosubstrate PKC-ε inhibitor. GDPβS abolished both initial and sustained contraction, whereas a Goαq antibody inhibited only initial contraction, implying that sustained contraction was dependent on activation of a distinct G protein. Sustained contraction induced by epidermal growth factor was inhibited by calphostin C, PKC-α,β,γ antibody, and a pseudosubstrate PKC-α inhibitor. Ca^{2+} (0.4 μM) induced an initial contraction in permeabilized muscle cells that was blocked by calmodulin and MLCK inhibitors and a sustained contraction that was blocked by calphostin C and a PKC-α,β,γ antibody. Thus initial contraction induced by Ca^{2+}, agonists, and growth factors is mediated by MLCK, whereas sustained contraction is mediated by specific Ca^{2+}-dependent and -independent PKC isozymes. G protein-coupled receptors are linked to PKC activation via distinct G proteins.

The initial Ca^{2+} transient induced by contractile agonists in smooth muscle is accompanied by Ca^{2+}/calmodulin (CaM)-dependent activation of myosin light chain (MLC) kinase (MLCK) and phosphorylation of Ser 19 on the 20-kDa regulatory light chain of myosin II (MLC20), leading to activation of the actin-activated myosin ATPase, interaction of actin and myosin, and muscle contraction (33, 34, 38). The initial Ca^{2+} transient is rapidly dissipated by extrusion of Ca^{2+} from the cell and uptake into intracellular Ca^{2+} stores, causing a rapid decline in MLCK activity that is accentuated by phosphorylation of MLCK via Ca^{2+}/CaM-dependent protein kinase II (35) and p21-activated kinase (31). Despite the rapid decline of intracellular Ca^{2+} concentration ([Ca^{2+}]) and MLCK activity to near-resting levels, MLC20 phosphorylation and muscle contraction are sustained, albeit at lower levels (9, 33, 34, 38).

Various mechanisms that can operate under reduced or resting Ca^{2+} levels have been proposed; all involve a regulated decrease in MLC phosphatase (MLCP) activity and assume that the increase in MLC20 phosphorylation reflects basal activity of MLCK or the activation of a Ca^{2+}-independent MLCK (13, 33, 36, 39). The contribution of other regulatory proteins, such as caldesmon and calponin, appears to be small (9, 22, 30, 38). A decrease in MLCP activity could result from 1) Rho kinase-mediated phosphorylation of the 130-kDa myosin-binding, regulatory subunit of MLCP (7, 12, 16, 36); 2) arachidonic acid-induced inactivation of the holoenzyme mediated by the atypical protein kinase C (PKC) isoform PKC-ζ (5, 37); and 3) PKC-dependent activation of an endogenous, 17-kDa inhibitor of MLCP, CPI-17 (3, 14, 19, 21). Inhibition of MLCP by Rho kinase or via a PKC-dependent mechanism may not be mutually exclusive. Recent studies (29) in intestinal smooth muscle have shown that contractile agonists initiate a G protein-dependent cascade involving sequential activation of RhoA, RhoA kinase, phospholipase D (PLD), and PKC, implying that Rho kinase could inhibit MLCP via a downstream, PKC-dependent mechanism.

Studies of Ca^{2+} sensitization have contributed greatly to discovery of the role of MLCP in maintaining MLC20 phosphorylation but are based on the assumption that sustained MLC20 phosphorylation and contraction reflect G protein-mediated enhancement or sensitization of Ca^{2+}-dependent mechanisms. It is equally plausible, as noted above, that sustained contraction may be mediated by G protein-dependent pathways that maintain MLC20 phosphorylation and attenuate dephosphorylation, independent of Ca^{2+}/CaM-dependent activation of MLCK.

The experimental design for measurement of Ca^{2+} sensitization has yielded conflicting results regarding
the role of PKC. Jensen et al. (10) have reported that in the presence of a fixed Ca\(^{2+}\) concentration (0.3 \(\mu M\)), the increase in contraction of rabbit vascular smooth muscle induced by agonists was either not affected or only partly inhibited by complete or partial (~70%) down-regulation of various PKC isozymes. Lee et al. (18), however, have reported that the increase in contraction in ferret aorta and portal vein smooth muscle was blocked by specific inhibitors of distinct PKC isozymes: the pattern of inhibition reflected the selective translocation of PKC-\(\epsilon\) in aortic muscle and PKC-\(\alpha\) and PKC-\(\beta\) in portal vein muscle.

In the present study, we adopted an experimental design that maintained the sequence of signaling events mediating the initial and sustained phases of contraction to examine the role of PKC. The results indicate that sustained contraction of intestinal circular and longitudinal muscle induced by agonists was independent of Ca\(^{2+}\) mobilization and reflected activation of PKC-\(\epsilon\) via a distinct G protein-dependent pathway. In contrast, sustained contraction induced by epidermal growth factor (EGF) and exogenous Ca\(^{2+}\) was mediated by Ca\(^{2+}\)-dependent PKC isozymes.

**MATERIALS AND METHODS**

**Preparation of dispersed intestinal smooth muscle cells.** Muscle cells were isolated separately from the circular and longitudinal muscle layers of guinea pig intestine by sequential enzymatic digestion, filtration, and centrifugation, as described previously (23, 28). Briefly, muscle strips were incubated at 31°C for 30 min in HEPES medium with type II collagenase (0.1%) and soybean trypsin inhibitor (0.1%). The partly digested strips were washed, and muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500-\(\mu M\) Nitex and centrifuged twice at 350 \(g\) for 10 min.

In experiments with blocking antibodies, the cells were permeabilized as described previously (23, 28) by incubation for 10 min with saponin (35 \(\mu g/ml\)) in a medium containing 1% BSA and (in mM) 20 NaCl, 100 KCl, 5 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 0.34 CaCl\(_2\), and 1 EGTA. The cells were centrifuged at 350 \(g\) for 5 min and resuspended in the same medium with 1.5 mM ATP-regenerating system (5 mM creatine phosphate and 10 U/ml creatine phosphokinase).

**Measurement of muscle cell contraction by scanning micrometry.** Contraction was measured in intact and permeabilized muscle cells by scanning micrometry as described previously (23, 24, 28). An aliquot containing 10\(^4\) cells/ml was added to 0.1 ml of medium containing CCK-8 (1 nM), neuromedin C (NMC; 100 nM), or EGF (10 nM), and the reaction was terminated at various intervals with 1% acrolein. The effect of PKC antibodies was determined in permeabilized muscle cells after preincubation for 1 h with different concentrations (1–1,000 ng/ml) of each antibody separately. The mean length of muscle cells treated with agonist was compared with the mean length of untreated cells, and contraction was expressed as percent decrease in mean cell length.

**Radioreceptor assay for inositol 1,4,5-trisphosphate in dispersed smooth muscle cells.** Inositol 1,4,5-trisphosphate (IP\(_3\)) mass was measured in intact cells as described previously (23, 28) using an assay system from Amersham. One milliliter of muscle cell suspension (10\(^6\) cells/ml) was incubated with Li\(^+\) for 10 min at 31°C, after which EGF (10 nM) or NMC (100 nM) was added for 30 s and the reaction was terminated with ice-cold 10% perchloric acid. After centrifugation for 10 min at 750 \(g\), the supernatant was extracted and IP\(_3\) content in the aqueous phase was measured. Results were expressed as picomoles per 10\(^6\) cells.

**Fig. 1.** Inhibition of CCK-stimulated initial contraction by neomycin and dimethyleicosadienoic acid (DEDA). Guinea pig intestinal circular (A) and longitudinal (B) smooth muscle cells were treated for 10 min with neomycin (50 \(\mu M\)) or DEDA (10 \(\mu M\)). Contraction was measured at intervals for 20 min in the presence and absence of each agent. Neomycin and DEDA inhibited initial contraction in circular and longitudinal muscle cells, respectively, but had no effect on sustained contraction. Contraction was expressed as %decrease (\(\Delta\%\)) in mean cell length from control. Values are means ± SE of 4 experiments.
Measurement of $[Ca^{2+}]_{i}$ in dispersed smooth muscle cells. $[Ca^{2+}]_{i}$ was measured in suspensions of muscle cells using the $Ca^{2+}$ fluorescent dye fura 2 as described previously (24, 26). Muscle cells were suspended in a medium containing (in mM) 10 HEPES, 125 NaCl, 5 KCl, 1 CaCl$_2$, 0.5 MgSO$_4$, 5 glucose, 20 taurine, 45 Na pyruvate, and 5 creatine and incubated with fura 2-AM (2 $\mu$M) for 20 min at 31°C. After centrifugation at 350 $g$ for 20 min, the cells were incubated in fura 2-free medium for immediate measurement of $Ca^{2+}$. Fluorescence was monitored at 510 nm, with excitation wavelengths alternating between 340 and 380 nm, and the measurements were corrected for autofluorescence of unloaded cells. An estimate of $[Ca^{2+}]_{i}$ was obtained from observed, maximal, and minimal fluorescence ratios as described previously (24, 26).

Measurement of MLCK activity. MLCK activity was measured by phosphorylation of a smooth muscle MLC substrate as described by Gilbert et al. (6). After treatment with agonist, the cells were homogenized in a medium containing (in mM) 50 KH$_2$PO$_4$, 4 EDTA, 15 dithiothreitol, 10 NaF, 1 phenylmethylsulfonyl fluoride (pH 6.8), 0.5% Triton X-100, and 10 $\mu$g/ml aprotinin and then centrifuged at 8,000 $g$ for 10 min. The supernatant was added to a mixture containing (in mM) 0.1 Ca$^{2+}$, 50 MOPS, 15 dithiothreitol, and 10 Mg acetate, 0.3 $\mu$M CaM, and 18 $\mu$M smooth muscle MLC. The reaction was initiated with 1 mM [y-$^{32}$P]ATP. Aliquots were spotted on Whatman filter paper, rinsed successively with 10% TCA, 4% pyrophosphate, 95% ethanol, and ethyl ether and then dried for measurement of radioactivity.

Identification of PKC isozymes in intestinal smooth muscle by Western blot. Cell homogenates were prepared from dispersed intestinal circular and longitudinal muscle cells separately and homogenized in a solution containing (in mM) 10 Tris · HCl (pH 7.5), 5 MgCl$_2$, 2 EDTA, 250 sucrose, 1 dithiothreitol, and 1 phenylmethylsulfonyl fluoride and 20 $\mu$g/ml leupeptin and 20 $\mu$g/ml aprotinin. The suspension was centrifuged at 100,000 $g$ for 30 min at 4°C, and the supernatant was collected as the cytosolic fraction. Pellets were resuspended, and proteins were extracted by incubation for 30 min in the homogenization buffer containing 1% Triton X-100 and 1% sodium cholate. The extract was centrifuged at 1,000 $g$ for 10 min, and the supernatant was collected as the particulate fraction. Solubilized membrane proteins (80–100 $\mu$g) were resolved by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After incubation in 5% nonfat dry milk to block nonspecific antibody binding, the blots were incubated with anti-rabbit IgG conjugated with

![Fig. 2. Time course of myosin light chain kinase (MLCK) activity in response to CCK-8. MLCK activity was measured in dispersed intestinal longitudinal muscle cells as described in MATERIALS AND METHODS. CCK-8 (1 nM) was added, and samples were obtained at intervals for 10 min. MLCK activity decreased rapidly, reverting to basal levels within 5–10 min. Identical results were obtained in intestinal circular muscle cells. Values are means ± SE of 4 experiments.](http://ajpgi.physiology.org/)

![Fig. 3. Inhibition of CCK-stimulated initial contraction by calmodulin (CaM) and MLCK inhibitors. Intestinal circular (A) and longitudinal (B) smooth muscle cells were treated for 10 min with the CaM inhibitor calmidazolium (1 $\mu$M) or the MLCK inhibitor KT-5926 (1 $\mu$M). Contraction was measured at intervals for 20 min in the presence and absence of each agent. Both inhibitors blocked initial contraction in circular and longitudinal muscle cells but had no effect on sustained contraction. Contraction was expressed as %decrease in mean cell length from control. Values are means ± SE of 4 experiments.](http://ajpgi.physiology.org/)
Materials. }^{32}P\text{ATP was obtained from NEN Life Sciences Products, HEPES from Research Organics (Cleveland, OH), and soybean trypsin inhibitor and collagenase (type II) from Worthington. Fura 2-AM was obtained from Molecular Probes, and calmidazolium, calphostin C, and chelerythrine chloride were from Calbiochem. Neomycin, KT-5926, and dimethyleicosadienoic acid (DEDA) were obtained from Bio- mol (Plymouth Meeting, PA). Gα<sub>q/11</sub> and PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were from Sigma Chemical. Selective N-myristoylated peptide inhibitors derived from pseudosubstrate sequences of PKC-α, PKC-αβγ, PKC-δ, and PKC-ε were gifts from Drs. D. A. Dartt and D. Zoukhri (Harvard Medical School).

RESULTS

Pathway mediating initial smooth muscle contraction induced by agonists. We (24, 26) have previously shown that Ca<sup>2+</sup> mobilization in circular muscle is mediated by IP<sub>3</sub>-dependent Ca<sup>2+</sup> release, whereas Ca<sup>2+</sup> mobilization in longitudinal muscle is initiated by phospholipase A<sub>2</sub> (PLA<sub>2</sub>)-dependent Ca<sup>2+</sup> influx, which triggers Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release via ryanodine receptors/Ca<sup>2+</sup> channels (15, 20, 26). Accordingly, the initial contraction induced by CCK-8 was blocked by the phosphoinositide inhibitor neomycin in circular muscle cells and by the PLA<sub>2</sub> inhibitor DEDA in longitudinal muscle cells (Fig. 1). The initial Ca<sup>2+</sup> transient in both muscle cell types was accompanied by an increase in MLCK activity that reached a peak within 30 s and declined rapidly to low levels within 2 min and to basal levels within 5–10 min (Fig. 2). Results similar to those shown in Fig. 2 for longitudinal muscle cells were obtained with circular muscle cells (20). The initial contraction in both cell types was inhibited by the CaM antagonist calmidazolium and by Ca<sup>2+</sup>/CaM-dependent MLCK inhibitor KT-5926 (Fig. 3). The effect of KT-5926 was concentration dependent with an IC<sub>50</sub> of 0.6 nM (Fig. 4). Treatment of permeabilized circular muscle cells with guanosine 5′-O-(2-thiodiphosphate) (GDPβS) abolished initial and sustained contraction, implying that both were G protein dependent (Fig. 5). In contrast, preincubation of permeabilized circular muscle cells with Go<sub>q/11</sub> antibody (10 μg/ml) for 60 min inhibited the initial contraction but had no effect on sustained contraction (Fig. 5).

Pathway mediating sustained contraction induced by agonists. A biphasic increase in DAG and PKC activity occurs in both muscle cell types consisting of an initial peak that is entirely dependent on phosphoinositide hydrolysis, followed by a sustained increase resulting from phosphatidylcholine hydrolysis by PLC and PLD (27). Nonselective PKC inhibitors as well as isozyme-selective inhibitors and antibodies were used to determine the involvement of PKC in sustained contraction. Chelerythrine (10 μM), which blocks the substrate binding site, abolished sustained contraction in both circular and longitudinal muscle cells but had no effect on initial contraction (Fig. 6). Identical results were obtained with calphostin C (1 μM), which blocks the

**Fig. 4. Concentration-dependent inhibition of CCK-stimulated contraction by MLCK and protein kinase C (PKC) inhibitors. Intestinal circular smooth muscle cells were treated for 10 min with various concentrations of the MLCK inhibitor KT-5926, and initial contraction in response to 1 nM CCK-8 was measured at 30 s (A). In separate experiments, the muscle cells were treated with various concentrations of the PKC inhibitor calphostin C, and sustained contraction was measured at 300 s (B). Contraction was expressed as % decrease in mean cell length from control. IC<sub>50</sub> of KT-5926 and calphostin C was 0.6 and 12 nM, respectively. Values are means ± SE of 3 experiments.**

**Fig. 5. Effects of guanosine 5′-O-(2-thiodiphosphate) (GDPβS) and Go<sub>q/11</sub> antibody (Ab) on CCK-stimulated contraction. Permeabilized intestinal circular muscle cells were incubated for 10 min with GDPβS or for 60 min with Go<sub>q/11</sub> before addition of 1 nM CCK-8. Initial contraction was measured at 30 s and sustained contraction at 300 s after addition of CCK-8. GDPβS inhibited initial (30 s) and sustained (300 s) contraction, whereas Go<sub>q/11</sub> antibody inhibited initial contraction only, suggesting involvement of a distinct G protein in sustained contraction. Contraction was expressed as % decrease in mean cell length from control. Values are means ± SE of 4 experiments. **P < 0.01 vs. control.**
DAG binding site; the effect of calphostin C was concentration dependent with an IC₅₀ of 12 nM (Fig. 4).

Preincubation of permeabilized circular muscle with a specific PKC-ε antibody (1 μg/ml) for 1 h abolished sustained contraction but had no effect on initial contraction, whereas preincubation with a common PKC-α,β,γ antibody (1 μg/ml) had no effect on initial or sustained contraction (Fig. 7). The IC₅₀ of PKC-ε antibody in inhibiting sustained contraction was 20 ng/ml (Fig. 7). Similar results were obtained with selective myristoylated pseudosubstrate PKC inhibitors in intact circular and longitudinal muscle cells. A selective PKC-ε inhibitor blocked sustained contraction (measured after 5 min of agonist stimulation) but had no effect on initial contraction (measured at 30 s), whereas selective PKC-α, PKC-δ, and PKC-α,β,γ inhibitors had no effect on sustained or initial contraction (Fig. 8).

Western blot analysis using a specific PKC-ε antibody and a common PKC-α,β,γ antibody showed that CCK-8 induced delayed translocation of PKC-ε and rapid translocation to the membrane of one or more of the following: PKC-α, PKC-β, or PKC-γ. Translocation of PKC isozymes attained a plateau within 5 min (Fig. 9). The pattern of translocation was similar in circular and longitudinal muscle cells.

Pathways mediating initial and sustained contraction induced by EGF and NMC. The effects of various inhibitors and PKC antibodies on contraction induced by EGF and NMC, the active COOH-terminal decapeptide of gastrin-releasing peptide, were examined in rabbit intestinal circular muscle cells. The response to CCK-8 in rabbit muscle cells was previously shown to be closely similar to that in guinea pig muscle cells (15, 29). EGF (10 nM) and NMC (100 nM) stimulated IP₃ formation (16.0 ± 3.2 and 10.0 ± 2.3 pmol/10⁶ cells, respectively), increased [Ca²⁺]ᵢ levels (388 ± 53 and 390 ± 20 nM, respectively), and induced contraction. Neomycin abolished the increase in IP₃ and [Ca²⁺]ᵢ induced by both peptides and inhibited contraction during the first 2 min (Fig. 10). Sustained contraction was not affected by neomycin but was inhibited by calphostin C (Fig. 10).

Sustained contraction induced by NMC was abolished by preincubation of permeabilized circular muscle cells for 1 h with PKC-ε antibody (1 μg/ml) but not with a common PKC-α,β,γ antibody (1 μg/ml). In contrast, sustained contraction induced by EGF was abolished by preincubation of permeabilized circular mus-
cle cells for 1 h with a common PKC-α,β,γ antibody (1 μg/ml) but not with a PKC-ε antibody (Fig. 11). A specific pseudosubstrate PKC-α inhibitor (1 μM) inhibited EGF-induced sustained contraction by 63.4 ± 3.7% (P < 0.001); the residual response reflected involvement of PKC-β and/or PKC-γ.

**Pathways mediating initial and sustained contraction induced by Ca²⁺.** Contraction induced by a near-maximal concentration of Ca²⁺ (0.4 μM) in permeabilized circular and longitudinal muscle cells rose to a peak within 30 s before declining to a lower sustained level (Fig. 12). The initial contraction was abolished by calmidazolium and KT-5926, implying that it was mediated by activation of Ca²⁺/CaM-dependent MLCK (Fig. 13). Unexpectedly, sustained contraction was not affected by calmidazolium or KT-5926 but was abolished by calphostin C (Fig. 13). Inhibition by calphostin C, which blocks the DAG binding site of PKC, implied that a high concentration of Ca²⁺ activated one or more phospholipases capable of generating DAG (17). Preincubation of permeabilized circular and longitudinal muscle cells with a common PKC-α,β,γ antibody abolished sustained contraction but had no effect on initial contraction; preincubation with a specific PKC-ε antibody had no effect on initial or sustained contraction (Fig. 12).

**DISCUSSION**

The experimental design adopted in the present study attempted to maintain the normal sequence of signaling events that mediate the initial and sustained phases of contraction in smooth muscle. Selective inhibitors were used in intact muscle cells and specific PKC and G protein antibodies in permeabilized muscle cells to demonstrate that agonist-stimulated, sustained contraction was independent of Ca²⁺ mobilization and reflected G protein-dependent activation of PKC-ε. Sustained contraction induced by EGF, however, reflected activation of one or more Ca²⁺-dependent PKC isoforms (PKC-α, PKC-β, and/or PKC-γ). Unexpectedly, sustained contraction induced by Ca²⁺ in permeabilized muscle cells was also mediated by PKC-α, PKC-β, and/or PKC-γ. The evidence on which these conclusions are based is summarized below.

Inhibition of agonist-stimulated Ca²⁺ mobilization by neomycin in intestinal circular muscle (26) and by DEDA in longitudinal muscle (26) abolished initial contraction.
contraction but had no effect on sustained contraction. Because neither Ca\(^{2+}\) release nor capacitative Ca\(^{2+}\) influx occurs under these conditions, the mechanism(s) responsible for sustained contraction did not require an increase in [Ca\(^{2+}\)]\(_i\) above resting levels. The lack of effect of DEDA on sustained contraction suggests that PLA\(_2\) products, such as arachidonic acid, are not involved in maintaining contraction (5, 33).

Suppression of Ca\(^{2+}\)/CaM-dependent MLCK activity while maintaining Ca\(^{2+}\) mobilization also abolished initial contraction but had no effect on sustained contraction. This implied that MLC\(_{20}\) phosphorylation by Ca\(^{2+}\)/CaM-dependent MLCK is not a prerequisite for sustained contraction. Sustained MLC\(_{20}\) phosphorylation by other kinases such as Ca\(^{2+}\)-independent MLCK (39) and RhoA kinase (1) could occur, amplified by inhibition of MLCP (12, 36).

Suppression of G protein activity by GDP\(\beta\)S abolished both initial and sustained contraction, whereas blockade of G\(_{q/11}\), which initiates the cascade that leads to Ca\(^{2+}\) mobilization (28) abolished initial contraction only, suggesting that distinct heterotrimeric and/or monomeric G proteins are involved in sustained contraction.

Sustained contraction induced by CCK-8 was abolished by 1) chelerythrine and calphostin C, which block the substrate- and DAG-binding sites of PKC, respectively, 2) a specific PKC-\(\epsilon\) antibody, and 3) a selective N-myristoylated pseudosubstrate peptide inhibitor of PKC-\(\epsilon\). The specific involvement of PKC-\(\epsilon\) in sustained contraction was corroborated by results obtained with NMC, which also interacts with a G protein-coupled receptor (11).

Sustained contraction induced by the growth factor EGF, however, was abolished by a common PKC-\(\alpha,\beta,\gamma\) antibody and partially inhibited by a selective myristoylated pseudosubstrate peptide inhibitor of PKC-\(\alpha\), implying that it was mediated by PKC-\(\alpha\) as well as by PKC-\(\beta\) and/or PKC-\(\gamma\). Sustained contraction induced by phorbol 12-myristate 13-acetate was also abolished by a PKC-\(\alpha,\beta,\gamma\) antibody but was not affected by a PKC-\(\epsilon\) antibody (K. S. Murthy, unpublished observations). Thus depending on the agonist, Ca\(^{2+}\)-dependent and -independent PKC isozymes can mediate sustained contraction.

**Fig. 10.** Effect of neomycin and calphostin C on epidermal growth factor (EGF)- and neuromedin C (NMC)-stimulated contraction. Intestinal circular smooth muscle cells were treated with 10 nM EGF (A) or 100 nM NMC (B) in the presence and absence of neomycin (50 \(\mu\)M) or calphostin C (1 \(\mu\)M). Contraction was measured at 30 s (initial contraction) and 300 s (sustained contraction). Neomycin partly inhibited initial contraction but had no effect on sustained contraction. Sustained contraction was abolished by calphostin C. Similar results were obtained with longitudinal muscle cells (data not shown). Contraction was expressed as % decrease in mean cell length from control. Values are means \(\pm\) SE of 3 experiments. **\(P < 0.01\) vs. control.

**Fig. 11.** Differential inhibition of EGF- and NMC-stimulated sustained contraction by PKC isozyme antibodies. Permeabilized intestinal circular smooth muscle cells were incubated for 60 min with a specific PKC-\(\epsilon\) antibody (1 \(\mu\)g/ml) or a common PKC-\(\alpha,\beta,\gamma\) antibody (1 \(\mu\)g/ml) before addition of EGF (10 nM) or NMC (100 nM); contraction was measured at 30 s (initial contraction) and 300 s (sustained contraction). PKC-\(\alpha,\beta,\gamma\) antibody inhibited sustained contraction induced by EGF (A), and PKC-\(\epsilon\) antibody inhibited sustained contraction mediated by NMC (B). Contraction was expressed as % decrease in mean cell length from control. Values are means \(\pm\) SE of 3 experiments. **\(P < 0.01\) vs. control.
Unexpectedly, a near-maximal concentration of Ca\(^{2+}\) (0.4 \(\mu\)M) induced an initial contraction mediated by Ca\(^{2+}\)/CaM-dependent MLCK, followed by a sustained contraction mediated by PKC. Sustained contraction was abolished by calphostin C and by a common PKC-\(\alpha,\beta,\gamma\) antibody but was not affected by a CaM antagonist or a MLCK inhibitor. This suggests that Ca\(^{2+}\)/CaM-dependent MLCK was inactive at high Ca\(^{2+}\) concentrations, possibly inactivated by other kinases, e.g., Ca\(^{2+}\)/CaM-dependent protein kinase II and/or p21-activated kinase. Inhibition by calphostin C implied that high Ca\(^{2+}\) concentrations activated one or more phospholipases capable of generating DAG and activating PKC. Previous studies (25) had shown that increasing [Ca\(^{2+}\)] in the absence of receptor activation stimulates PKC activity.

Thus sustained contraction induced by activation of G protein-coupled receptors (CCK and NMC) and receptor tyrosine kinases (EGF) or by high levels of Ca\(^{2+}\) is mediated variously by Ca\(^{2+}\)-dependent or -independent PKC isozymes. Studies designed to elicit evidence of Ca\(^{2+}\) sensitization, however, have yielded contradictory results regarding the involvement of PKC in sustained muscle contraction (8, 10, 18, 37). In these studies, [Ca\(^{2+}\)] is clamped below (0.01 \(\mu\)M) or above (0.3–30 \(\mu\)M) resting [Ca\(^{2+}\)] levels (0.1 \(\mu\)M) to probe the effects of various agents (agonists, GTP\(_{\gamma}\)S, active phorbol esters, DAG analogs, and MLCP inhibitors). Morgan and co-workers (8, 9, 18, 22) have studied extensively the response of arterial and portal vein single smooth muscle cells in the ferret. Their studies (8, 9, 18, 22) disclosed the existence of a slowly developing, agonist-stimulated, PKC-dependent contraction that was mediated by PKC-\(\epsilon\) in arterial muscle cells at resting Ca\(^{2+}\) levels and by PKC-\(\alpha\) and/or PKC-\(\beta\) in portal vein muscle cells at higher Ca\(^{2+}\) levels. The involvement of distinct PKC isozymes reflected the predominant expression and translocation of these isozymes in the two types of smooth muscle. A similar tissue-specific translocation of PKC isozymes was also reported by Sohn et al. (32) in smooth muscle cells of the cat esophageal body and the lower esophageal sphincter. Contraction induced by a DAG analog in sphincteric circular muscle, which resembles intestinal circular muscle in its signaling properties, was mediated by a splice variant of PKC-\(\beta\) (PKC-\(\beta\)II), whereas contraction in esophageal muscle was mediated by PKC-\(\epsilon\); in the latter tissue, agonist-stimulated contrac-

![Fig. 12. Inhibition of Ca\(^{2+}\)-stimulated sustained contraction by PKC-\(\alpha,\beta,\gamma\) antibody. Permeabilized intestinal circular (A) and longitudinal (B) smooth muscle cells were exposed to 400 nM Ca\(^{2+}\) alone or after a 60-min incubation with a specific PKC-\(\epsilon\) antibody (1 \(\mu\)g/ml) or a common PKC-\(\alpha,\beta,\gamma\) antibody (1 \(\mu\)g/ml). Contraction was measured at intervals for 20 min in the presence or absence of antibodies. PKC-\(\alpha,\beta,\gamma\) antibody inhibited sustained contraction but had no effect on initial contraction. PKC-\(\epsilon\) antibody had no effect on initial or sustained contraction. Contraction was expressed as % decrease in mean cell length from control. Values are means ± SE of 3 experiments.](image1)

![Fig. 13. Effect of various inhibitors on Ca\(^{2+}\)-stimulated initial and sustained contraction. Permeabilized intestinal circular (A) and longitudinal (B) muscle cells were treated for 10 min with each agent before raising the Ca\(^{2+}\) concentration to 400 nM. Initial contraction was measured at 30 s, and sustained contraction was measured at 300 s in the presence or absence of each inhibitor. The CaM and MLCK inhibitors blocked initial contraction, whereas calphostin C blocked sustained contraction in both muscle cell types. Contraction was expressed as % decrease in mean cell length from control. Values are means ± SE of 3 experiments.](image2)
activation of RhoA, RhoA kinase, and PLD. Conversion of G protein (G13)-dependent activation of RhoA lead to inhibition of MLC20 phosphatase and enhancement of phosphorylation of MLC20 at Ser19 (1), as well as inhibition of RhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. Mol Biol Cell 8: 2437–2447, 1997.


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