

## Role of various kinases in muscarinic M3 receptor-mediated contraction of longitudinal muscle of rat colon

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### Abstract

The longitudinal muscle layer in gut is the functional opponent to the circular muscle layer during peristalsis. Differences in innervation of the layers allow for the contraction of one layer concurrently with the relaxation of the other, enabling the passage of gut contents in a controlled fashion. Differences in development have given the cells of the two layers differences in receptor populations, membrane lipid handling, and calcium handling profiles/behaviors. The contractile activity of the longitudinal muscle is largely mediated by cholinergic neural input from myenteric plexus. Activation of muscarinic receptors leads to rapid activation of several kinases including MLC kinase, ERK1/2, CaMKII and Rho kinase. Phosphorylation of myosin light chain (MLC<sub>20</sub>) by MLC kinase (MLCK) is a prerequisite for contraction in both circular and longitudinal muscle cells. In rat colonic longitudinal muscle strips, we measured muscarinic receptor-mediated contraction following incubation with kinase inhibitors. Basal tension was differentially regulated by Rho kinase, ERK1/2, CaMKII and CaMKK. Selective inhibitors of Rho kinase, ERK1/2, CaMKK/AMPK, and CaMKII each reduced carbachol-induced contraction in the innervated muscle strips. These inhibitors had no direct effect on MLCK activity. Thus unlike previously reported for isolated muscle cells where CaMKII and ERK1/2 are not involved in contraction, we conclude that the regulation of carbachol-induced contraction in innervated longitudinal muscle strips involves the interplay of Rho kinase, ERK1/2, CaMKK/AMPK, and CaMKII.

**Key words:** smooth muscle, gastrointestinal tract, motility

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## Introduction

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Gastrointestinal motility requires coordinated activity of the two muscular layers of the gut, the circular muscle layer and the longitudinal muscle layer (1–4). The circular muscle layer consists of muscle cells oriented around the circumference of the gut lumen, forming adjacent rings of muscle that extend throughout the gut. Upon local activation of these cells, the circular muscle tissue contracts, and these muscular rings decrease their diameter and therefore the caliber of the gut lumen. The longitudinal muscle layer is comprised of cells oriented along the long axis of the gut, parallel to the overall direction of the movement of contents. Upon local activation of the peristaltic reflex, contraction of the longitudinal muscle causes the gut to shorten while the relaxation of the circular muscle layer causes an increase in the diameter of the lumen. Thus, the two muscular layers are not normally activated at the same location simultaneously (5–7); however, when these opposing actions occur in the longitudinal and circular muscle layers at contiguous locations, the contents of the lumen can be propelled distally in a controlled fashion. Functional differences are evident between the two muscle layers and reflect distinct receptor expression, calcium handling, and signaling pathways involved in muscle contraction (7–14).

Muscle activity is driven by the interaction of the proteins actin and myosin, which facilitates the inherent ATPase activity of the heavy chains of myosin. The phosphorylation of the 20 kDa myosin regulatory light chain (MLC<sub>20</sub>) at a serine residue at position 19 is sufficient to cause cross-bridge formation and cycling, ATP hydrolysis, and muscle contraction (15, 16). Myosin light chain kinase (MLCK) is a dedicated serine-threonine kinase that has MLC<sub>20</sub> as its sole substrate. Upon binding with the calcium-calmodulin complex, MLCK phosphorylates MLC<sub>20</sub> at serine 19 (15–17). Myosin light chain phosphatase (MLCP) is the opposing enzyme to MLCK, removing the phosphate from Serine 19 on MLC<sub>20</sub>. It is a trimer composed of protein phosphatase 1c-delta, the myosin phosphatase target subunit 1 (MYPT1), and a small 20kDa subunit MP20. MYPT1 targets the phosphatase subunit to MLC<sub>20</sub>, while increasing the specificity of PP1c-delta through a conformational change that occurs when bound to MYPT1. The MP20 subunit is of unknown function (18, 19).

Many studies have shown that MLC<sub>20</sub> phosphorylation can be regulated, either directly or through the regulation of MLCP activity and associated proteins, by several other kinases including Rho kinase, ERK1/2, CaMKII, PAK and CaMKK (20–35). This regulation is important during the agonist-induced sustained contraction as MLCK activity is transient and in synchronization with the transient nature of increase in Ca<sup>2+</sup> levels (16, 36, 37). The activity of MLCK itself can be affected by its phosphorylation at several site(s); kinases that phosphorylate can increase or decrease MLCK activity, and may do so by either altering the rate of enzyme activity or the affinity of MLCK for Ca-CaM (38–42).

Our previous studies have shown that in both circular and longitudinal intestinal smooth muscle, contraction in response to G protein-coupled receptor agonists is biphasic (36, 37). However, the mechanisms that mobilize Ca<sup>2+</sup> for initial contraction and regulate MLCP activity for sustained contraction are distinct in these types of muscle (8, 13). The aim of the present study is to characterize the role of various kinases that are activated in response to contractile agonists in the regulation of MLCK activity and muscle contraction in response to muscarinic receptor activation in innervated longitudinal muscle strips. Our results demonstrate a distinct pattern of regulation. Similar to studies in isolated muscle cells, inhibition of both Rho kinase and ERK1/2 leads to diminished contraction in innervated muscle strips, but unlike isolated cells, inhibition of CaMKK/AMPK and CaMKII also diminished contraction.

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## Materials and Methods

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### *Materials*

Y27632, PD98059, STO-609, and KN62 were purchased from Calbiochem (La Jolla, CA). 15% Tris-HCl Ready Gels and DC Protein Assay Kit were products of Bio-Rad (Hercules, CA), Myelin Basic Protein was purchased from Upstate Biotechnology (now Millipore, Billerica, MA) and [ $\gamma$ - $^{32}$ P]ATP from Perkin Elmer Life Sciences (Boston, MA). Antibody for MLCK and protein A/G agarose beads are products of Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Sprague-Dawley Rats were purchased from Charles River Laboratories and housed in the animal facility of the Division of Animal Resources, Virginia Commonwealth University. All procedures followed guidelines of and were in accordance with the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

### *Animal Preparation*

Rats were euthanized by CO<sub>2</sub> asphyxiation under approved protocols. The colon was dissected out, emptied of contents and placed in a warmed (37°C) oxygenated Krebs solution of the following composition (in mM): 118 NaCl, 4.75 KCl, 1.19 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 2.54 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 11 mM glucose (pH 7.4). 2–3 cm sections of colon were removed and threaded onto a glass rod, where the longitudinal muscle/myenteric plexus (LM-MP) was removed by radial abrasion with a lab wipe. The resultant strip of muscle was freed of excess fat and mesenteric attachments and held in oxygenated Krebs buffer until use for tension recording or molecular assay.

### *Muscle Strip Preparation*

Strips destined for recordings of contractile behavior were tied at both ends with surgical silk so that one end had a simple loop for attachment to a glass hook and the other end had a length of silk tied to a brass ring. The strip was then placed in a vertical orientation with the loop secured to a glass hook and the brass ring to a Model FT03C Force Transducer (Grass Technologies, Quincy, MA). An organ bath (Radnoti, Monrovia, CA) was raised to submerge the strip in 5 mL of continuously oxygenated and warmed Krebs solution. Force recordings were amplified by a 15A12 model amplifier (contained within a Model 15LT Amplifier System), relayed to a PVA-16 Polyview Adaptor Unit (A/D-D/A converter), and displayed/stored by a PC running Polyview Version 1.3 (Grass Technologies, Quincy, MA). Force was recorded in grams.

Strips destined for molecular assay were treated as those intended for recordings and the time scales for inhibitor incubation and agonist exposure were identical. Following prescribed incubation times, tissue strips were submerged in a bath of Krebs with 1  $\mu$ M carbachol. After 60 seconds of immersion in carbachol/Krebs (corresponding to a normal vigorous response seen in strips), strips were flash frozen in liquid nitrogen and placed into TPER (Tissue Protein Extraction Reagent, Pierce, Rockford, IL) or lysis buffer with composition 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 10 mM sodium pyrophosphate. In addition, a protease/phosphatase cocktail (100  $\mu$ g/mL PMSF, 10  $\mu$ g/mL leupeptin, 30 mM sodium fluoride and 3 mM sodium vanadate) was added at a concentration of 2  $\mu$ L/mL. Tissue was homogenized and solubilized in the above solutions. Following centrifugation at 20000 g for 15 min at 4°C, the protein concentration of the supernatant was assessed with a DC protein assay kit. These supernatant lysates were stored at –80°C until needed for immunokinase assay.

### *Isometric force measurement*

Force experiments were conducted in the following manner. Following hanging of the strip and submersion in the organ bath, strips were subjected to approximately 1 gram of pre-tension via the mounting rack-and-pinion. Strips were allowed to equilibrate for no less than 30 minutes before experiments were conducted and data collected. Exposure to inhibitors, blockers, and carbachol occurred within the organ bath. Concentrations were appropriate and in agreement with current literature and are noted in the results. Following an experiment, strip data were reviewed and analyzed from within the Polyview software suite. One way ANOVA and paired *t*-tests were conducted in GraphPad (GraphPad Software, La Jolla, CA), and significance set at  $P < 0.05$ . All tests of significance were done by comparison of raw data between control and experimental groups.

### *Data Analysis*

Contractile data was viewed from three perspectives as to the effects of kinase inhibition: changes to basal tone upon kinase inhibitor administration, peak contractile amplitude following agonist exposure, and as area under the curve measurement. The latter was used to quantify the contraction as viewed during the first two minutes of the contraction in an effort to determine differences in force development/decay. All numerical values are expressed as mean  $\pm$  S.E.M.

Basal tension was measured as the mean tension during a 3 minute period following at least 30 min of equilibration (control conditions) or 10 min of inhibitor incubation (basal recording obtained during the interval preceding carbachol administration). Such measurements were made in multiple strips from multiple animals, and paired *t*-tests conducted to determine a significant effect. Peak contraction (amplitude) was defined to be the greatest amplitude of tone above basal during the two minute period following agonist administration. This two minute period is taken to begin when the contractile response began to rise from basal value. Area under curve (AUC) for first two minutes of exposure reflects the development and maintenance of the tension and was expressed as gram-seconds.

### *Kinase Assay*

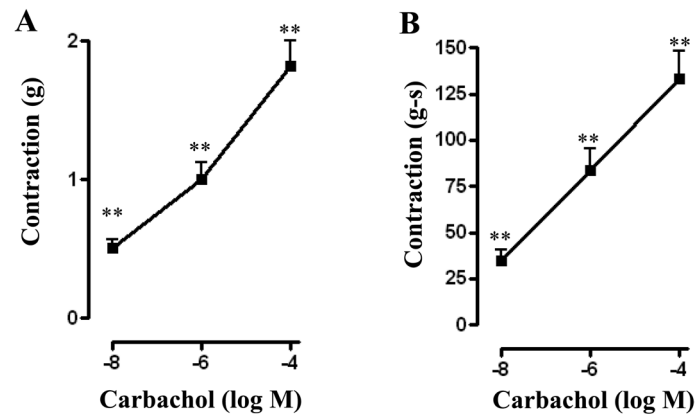
Activity of MLCK activity was measured by an immunokinase assay as described previously (37, 43, 44). One hundred  $\mu\text{g}$  of protein was transferred from the supernatant of prepared tissue sample to a designated tube, 1  $\mu\text{g}$  of MLCK goat antibody was added, and this mixture incubated for 2 hours. Protein A/G agarose beads were added to each sample, and the mixture again incubated at 4°C overnight. Following centrifugation, supernatants were withdrawn and the beads/protein washed with lysis buffer three times. The bead/protein pellets were resuspended in kinase buffer with composition (in mM): 50  $\text{KH}_2\text{PO}_4$ , 15 dithiothreitol (DTT), 10 NaF, 1 PMSE, 0.5% Triton X-100 and 10  $\mu\text{g}/\text{ml}$  aprotinin. Twenty microliters of supernatant were added to a mixture containing (in mM) 0.1  $\text{Ca}^{2+}$ , 50 Mops, 15 DTT, 10 magnesium acetate, and 0.3  $\mu\text{M}$  calmodulin and 18  $\mu\text{M}$  smooth-muscle  $\text{MLC}_{20}$ . The reaction was initiated with 1 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Aliquots were spotted on Whatman filter paper, rinsed successively with 75 mM phosphoric acid, 95% (v/v) ethanol and 100% (v/v) diethyl ether and were dried for measurement of radioactivity.

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## Results

### *Muscarinic m3 receptor-mediated contraction*

Longitudinal muscle strips exposed to carbachol (CCh) at concentrations of 10 nM, 1  $\mu\text{M}$ , and 100  $\mu\text{M}$  demonstrated contraction in a concentration-dependent manner and the maximal response was obtained with



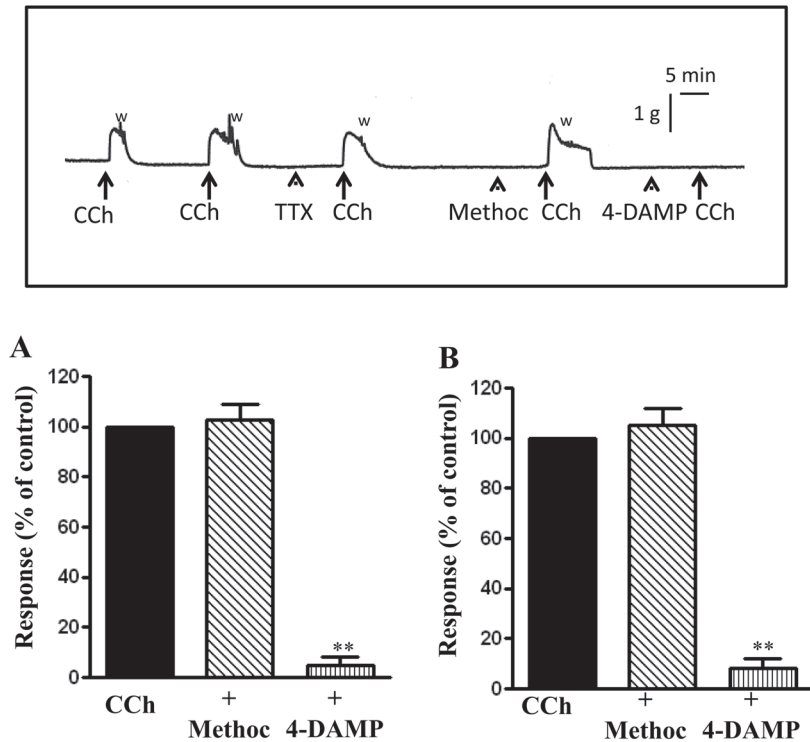
**Fig. 1.** Contractile response of longitudinal muscle strips to carbachol. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, different concentrations of carbachol (CCh, 10 nM to 100  $\mu$ M) was added. Contractile response with maximum force was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Carbachol elicited dose-dependent increase in both peak and total contraction. Values are means  $\pm$  SEM of 6–7 experiments and each experimental value was derived from several strips.  $**P < 0.05$  significant contraction.

100  $\mu$ M of CCh. Peak contractile responses were  $0.51 \pm 0.06$  grams at 10 nM CCh ( $n=40$ ),  $1.00 \pm 0.12$  grams at 1  $\mu$ M CCh ( $n=50$ ) and  $1.80 \pm 0.18$  grams at 100  $\mu$ M CCh ( $n=54$ ). Contractile responses calculated as area under curve (AUC) for first 2 minutes were  $34.44 \pm 5.84$  gram-seconds at 10 nM CCh ( $n=34$ ),  $83.62 \pm 11.35$  gram-seconds at 1  $\mu$ M CCh ( $n=42$ ), and  $133.25 \pm 14.67$  gram-seconds at 100  $\mu$ M CCh ( $n=45$ ) (Fig. 1A and 1B).

Repeated measurements of peak contraction and area under the curve in response to 1  $\mu$ M CCh were conducted on strips following wash for 15 min in Krebs buffer and contractions were calculated as percentage of initial contraction before wash. There were no significant differences in either peak contraction or AUC with repeated measurements. Following a 15 min incubation after initial contraction, peak contraction was  $98.29\% \pm 3.99\%$  ( $n=13$ , 5 animals) and the AUC for 2 min was  $102.1\% \pm 1.88\%$  ( $n=13$ , 5 animals) of the initial contraction (Fig. 2A and 2B).

Smooth muscle expresses both m2 and m3 muscarinic receptors (45–48). As shown previously in circular muscle, CCh-induced contraction in longitudinal muscle is mediated primarily by muscarinic m3 receptors. Muscle strips were pretreated with m2 receptor antagonist, methoctramine (1  $\mu$ M) or m3 receptor antagonist 4-DAMP (1  $\mu$ M) and the response to 1  $\mu$ M CCh was measured. Incubation with methoctramine caused no significant changes to peak contraction or AUC in response to CCh ( $P > 0.05$ ,  $n=7$  from 4 animals). Incubation with 4-DAMP, however, abolished responses to CCh (Fig. 2). These results indicate that contraction in response to CCh is mediated mainly by activation of m3 receptors.

To further examine the smooth muscle muscarinic m3 receptors, muscle strips were incubated with 1  $\mu$ M tetrodotoxin (TTX), a  $\text{Na}^+$  channel blocker, and the contractile response to different concentrations of CCh was measured. Consistent with the blockade of CCh-induced contraction with m3 receptor antagonist, incubation of muscle strips with 1  $\mu$ M tetrodotoxin (TTX), had no significant changes to peak contraction or AUC in response to different concentrations of CCh ( $P > 0.05$ ,  $n=7$  from 4 animals). Peak contractions in the presence of TTX exposure were  $102.8\% \pm 5.98\%$  of the control value at 1  $\mu$ M CCh ( $n=7$ ),  $113.2 \pm 8.6\%$  at 10  $\mu$ M CCh ( $n=7$ ), and  $110.5 \pm 9.1\%$  at 100  $\mu$ M CCh ( $n=7$ ) and the AUC were  $112.1 \pm 7.3\%$  of the control value at 1  $\mu$ M,



**Fig. 2.** Contractile response to carbachol is mediated via m3 receptors. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with 1  $\mu$ M of the m2 receptor antagonist methoctramine or 1  $\mu$ M of the m3 receptor antagonist 4-DAMP and then with CCh (1  $\mu$ M). Contractile response with maximum force was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Carbachol elicited contraction was selectively blocked by 4-DAMP. Values are means  $\pm$  SEM of 4 experiments and each experimental value derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction. The inset illustrates an original tracing showing abolition of carbachol-induced contraction by 1  $\mu$ M 4-DAMP but not by 1  $\mu$ M tetrodotoxin (TTX) or 1  $\mu$ M methoctramine. “W” indicates when the preparation was washed with fresh Kreb’s buffer after peak contraction. Horizontal bar indicates time in minutes and the vertical bar illustrates force in grams.

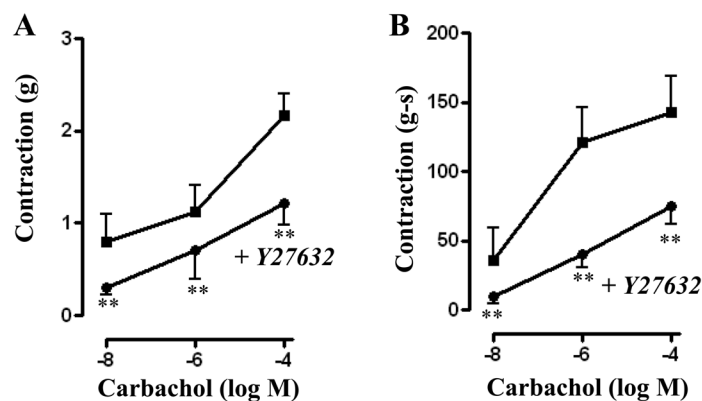
110.4  $\pm$  4.5% at 10  $\mu$ M and 97.7  $\pm$  8.3% at 100  $\mu$ M CCh ( $n$ =7).

### Regulation of CCh-induced contraction by kinases

Previous studies have shown that activation of m3 receptors leads to stimulation of various kinases such as Rho kinase, ERK1/2, CaMKII and AMPK and these kinases in turn, as shown in isolated cells, regulate phosphorylation of MLC<sub>20</sub> levels (37, 43, 44, 49). To examine the role of these kinases, muscle strips were pre-incubated with inhibitors of Rho kinase (Y27632), ERK1/2 (PD98059), CaMKII (KN62) or CaMKK (STO-609) and the response to CCh was measured.

#### 1) Regulation by Rho kinase

Treatment of muscle strips with Rho kinase inhibitor Y27632 (10  $\mu$ M) for 10 min led to a significant decrease in basal tone from 0.82  $\pm$  0.07 grams to 0.73  $\pm$  0.06 grams ( $P$ <0.01,  $n$ =15). Y27632 also significantly reduced contraction in response to different concentrations of CCh. Peak contractions (in grams) for control



**Fig. 3.** Effect of Rho kinase inhibitor, Y27632, on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective Rho kinase inhibitor Y27632 (10  $\mu$ M) and then with different concentrations of CCh (10 nM to 100  $\mu$ M). Contractile response with maximum force was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Values are means  $\pm$  SEM of 4 experiments and each experimental value was derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.

versus with Y27632 were  $0.80 \pm 0.30$  versus  $0.30 \pm 0.08$  ( $P$ <0.05,  $n$ =4) at 10 nM CCh;  $1.13 \pm 0.28$  versus  $0.71 \pm 0.19$  ( $P$ <0.01,  $n$ =15) for 1  $\mu$ M CCh; and  $2.17 \pm 0.38$  versus  $1.21 \pm 0.23$  ( $P$ <0.05,  $n$ =16) for 100  $\mu$ M (Fig. 3A). A similar inhibition of contraction was also observed when AUC was calculated and the values (in gram-seconds) were:  $35.54 \pm 24.26$  versus  $9.50 \pm 5.22$  ( $P$ <0.05,  $n$ =3) for 10 nM CCh;  $120.8 \pm 25.15$  versus  $39.90 \pm 9.42$  ( $P$ <0.01,  $n$ =10) for 1  $\mu$ M CCh; and  $142.7 \pm 26.03$  versus  $74.87 \pm 13.31$  ( $P$ <0.01,  $n$ =15) for 100  $\mu$ M (Fig. 3B).

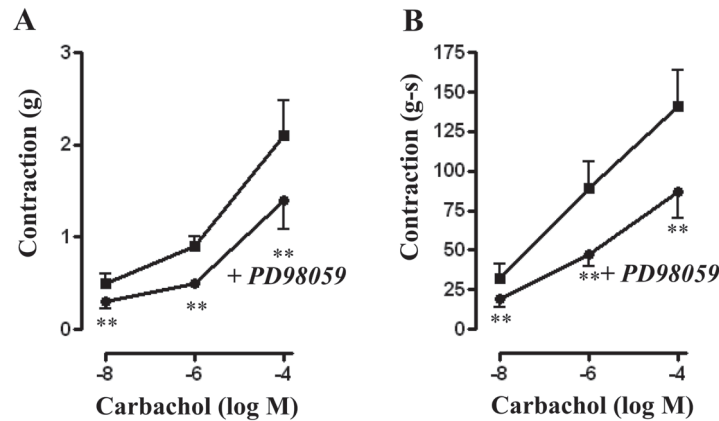
## 2) Regulation by ERK1/2

Treatment of muscle strips with ERK1/2 inhibitor PD98059 (10  $\mu$ M) for 10 min had no significant effect on basal tone ( $0.79 \pm 0.06$  grams versus  $0.81 \pm 0.05$  grams with PD98059). PD98059, however, significantly reduced contraction in response to different concentrations of CCh. Peak contractions (in grams) for control versus with PD98059 were  $0.47 \pm 0.13$  versus  $0.28 \pm 0.08$  ( $P$ <0.05,  $n$ =6) at 10 nM CCh;  $0.86 \pm 0.13$  versus  $0.49 \pm 0.09$  ( $P$ <0.01,  $n$ =9) for 1  $\mu$ M CCh; and  $2.06 \pm 0.38$  versus  $1.40 \pm 0.32$  ( $P$ <0.001,  $n$ =9) for 100  $\mu$ M (Fig. 4A). A similar inhibition of contraction was also observed when AUC was calculated and the values (in gram-seconds) were  $31.22 \pm 9.69$  versus  $18.78 \pm 5.57$  ( $P$ >0.05,  $n$ =7) for 10 nM CCh;  $87.82 \pm 17.61$  versus  $47.16 \pm 6.56$  ( $P$ <0.01,  $n$ =7) for 1  $\mu$ M CCh; and  $141.3 \pm 23.86$  versus  $87.66 \pm 16.69$  ( $P$ <0.01,  $n$ =9) for 100  $\mu$ M CCh (Fig. 4B).

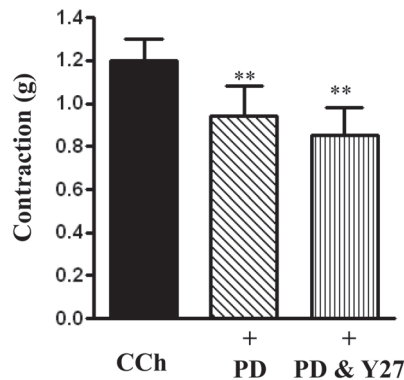
The inhibitory effect of Y27632 in combination with PD98059 was not significantly greater than the effect obtained with PD98059 alone:  $1.17 \pm 0.19$  grams for 1  $\mu$ M CCh,  $0.96 \pm 0.14$  grams CCh plus PD98059 alone ( $P$ <0.05),  $0.85 \pm 0.13$  grams for CCh plus PD98059 and Y27632 ( $P$ <0.05) (Fig. 5).

## 3) Regulation by CaMKK

Treatment of muscle strips with CaMKK inhibitor STO-609 (10  $\mu$ M) for 10 min had no significant effect on basal tone ( $0.76 \pm 0.10$  grams versus  $0.74 \pm 0.10$  grams with STO-609). STO-609, however, significantly reduced contraction in response to different concentrations of CCh. Peak contractions (in grams) for control versus with STO-609 were  $0.51 \pm 0.16$  versus  $0.32 \pm 0.14$  ( $P$ <0.05,  $n$ =6) at 10 nM CCh;  $1.31 \pm 0.20$  versus  $0.81 \pm 0.15$  ( $P$ <0.01,  $n$ =23) for 1  $\mu$ M CCh; and  $1.96 \pm 0.37$  versus  $1.22 \pm 0.20$  ( $P$ <0.05,  $n$ =16) for 100  $\mu$ M (Fig. 6A). A similar inhibition of contraction was also observed when AUC was calculated and the values (in gram-seconds)



**Fig. 4.** Effect of ERK1/2 inhibitor PD98059 on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective ERK1/2 inhibitor PD98059 (10  $\mu$ M) and then with different concentrations of CCh (10 nM to 100  $\mu$ M). Contractile response with maximum force was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Values are means  $\pm$  SEM of 4 experiments and each experimental value derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.

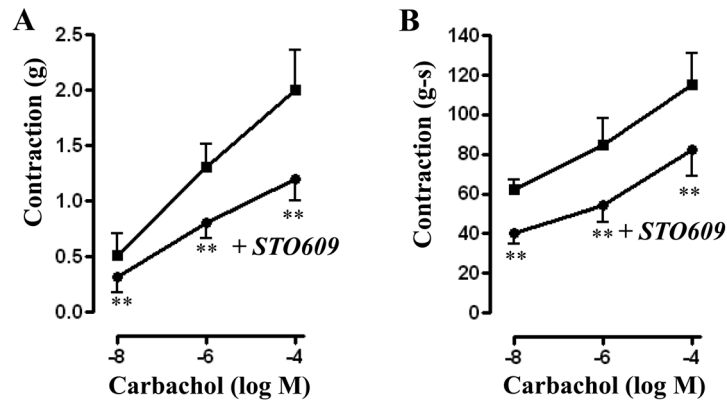


**Fig. 5.** Effect of PD98059 and Y27632 in combination on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective ERK1/2 inhibitor PD98059 (10  $\mu$ M) alone or in combination with Rho kinase inhibitor Y27632 (10  $\mu$ M) and then with CCh (1  $\mu$ M). Contractile response was measured as peak contraction. Values are means  $\pm$  SEM of 4–7 experiments and each experimental value was derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.

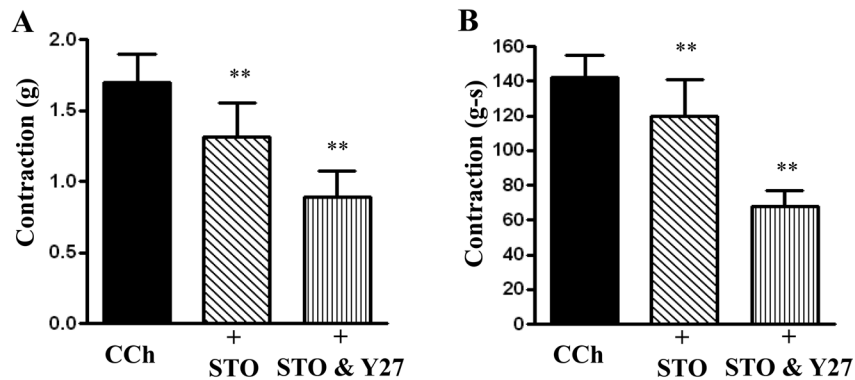
were  $62.64 \pm 5.40$  versus  $40.46 \pm 4.61$  ( $P$ <0.01,  $n=7$ ) for 10 nM CCh;  $85.42 \pm 13.77$  versus  $54.67 \pm 7.92$  ( $P$ <0.01,  $n=15$ ) for 1  $\mu$ M CCh; and  $114.6 \pm 16.23$  versus  $81.29 \pm 13.01$  ( $P$ <0.01,  $n=9$ ) for 100  $\mu$ M CCh (Fig. 6B).

The inhibitory effect of Y27632 in combination with STO-609 was significantly greater than the effect obtained with STO-609. Peak contractions were  $1.57 \pm 0.32$  grams for CCh alone,  $1.31 \pm 0.24$  grams for CCh plus STO-609 ( $P$ <0.05), and  $0.89 \pm 0.18$  grams for CCh plus STO-609 and Y27632 (Fig. 7A). For AUC the values (in grams-seconds) were  $142.6 \pm 13.86$  for CCh alone,  $122.4 \pm 26.22$  for CCh plus STO-609 ( $P$ <0.05,





**Fig. 6.** Effect of CaMKK inhibitor, STO-609 on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective CaMKK inhibitor STO-609 (10  $\mu$ M) and then with different concentrations of CCh (10 nM to 100  $\mu$ M). Contractile response with maximum force was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Values are means  $\pm$  SEM of 4–6 experiments and each experimental value derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.

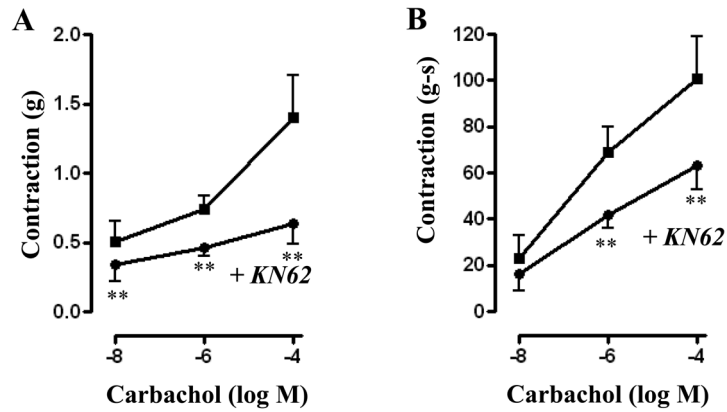


**Fig. 7.** Effect of STO-609 and Y27632 in combination on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective CaMKK inhibitor STO-609 (10  $\mu$ M) alone or in combination with Rho kinase inhibitor Y27632 (10  $\mu$ M) and then with CCh (1  $\mu$ M). Contractile response was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Values are means  $\pm$  SEM of 8 experiments and each experimental value derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.

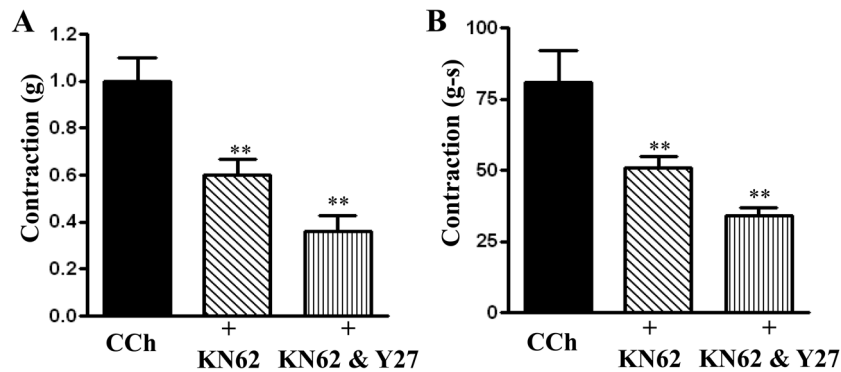
$n=7$ ), and  $68.36 \pm 9.72$  for CCh plus STO-609 and Y27632 ( $P$ <0.05,  $n=7$ ) (Fig. 7B). Thus, there is a significant ( $P$ <0.05 for both peak and AUC contraction) additive effect of CaMKK inhibition and Rho Kinase inhibition on both peak contraction and AUC.

#### 4) Regulation by CaMKII

Treatment of muscle strips with CaMKII inhibitor KN-62 (10  $\mu$ M) for 10 min caused a significant decrease in basal tone from  $0.61 \pm 0.07$  grams to  $0.53 \pm 0.07$  grams ( $P$ <0.05). KN-62 also significantly reduced

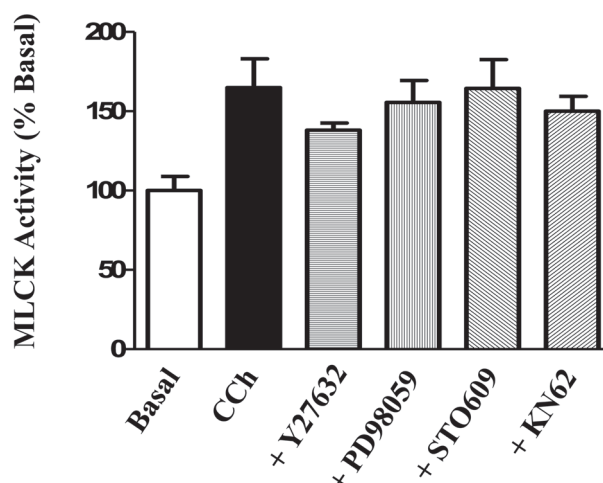


**Fig. 8.** Effect of CaMKII inhibitor, KN62 on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective CaMKII inhibitor KN62 (10  $\mu$ M) and then with different concentrations of CCh (10 nM to 100  $\mu$ M). Contractile response was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Values are means  $\pm$  SEM of 4 experiments and each experimental value derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.



**Fig. 9.** Effect of KN62 and Y27632 in combination on carbachol-induced contraction. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min with the selective CaMKII inhibitor KN62 (10  $\mu$ M) alone or in combination with Rho kinase inhibitor Y27632 (10  $\mu$ M) and then with CCh (1  $\mu$ M). Contractile response was measured as peak contraction (A) and total response for first 2 min, measured as area under curve (AUC), was considered as total contraction (B). Values are means  $\pm$  SEM of 8 experiments and each experimental value derived from several strips. \*\* $P$ <0.05 significant inhibition of CCh-induced contraction.

contraction in response to different concentrations of CCh. Peak contractions (in grams) for control versus with KN62 were  $0.52 \pm 0.14$  versus  $0.34 \pm 0.12$  ( $P$ <0.05,  $n=7$ ) at 10 nM CCh;  $0.74 \pm 0.10$  versus  $0.46 \pm 0.05$  ( $P$ <0.01,  $n=13$ ) at 1  $\mu$ M CCh; and  $1.35 \pm 0.32$  versus  $0.64 \pm 0.15$  ( $P$ <0.05,  $n=7$ ) at 100  $\mu$ M (Fig. 8A). A similar inhibition of contraction was also observed when AUC was calculated and the values (in gram-seconds) were  $22.13 \pm 10.10$  versus  $16.14 \pm 7.43$  for 10 nM;  $69.38 \pm 11.85$  versus  $42.11 \pm 5.99$  ( $P$ <0.05,  $n=12$ ) for 1  $\mu$ M; and  $100.7 \pm 18.58$  versus  $63.46 \pm 10.27$  ( $P$ <0.05,  $n=5$ ) for 100  $\mu$ M (Fig. 8B).



**Fig. 10.** Effect of kinase inhibitors on carbachol-induced MLCK activity. Longitudinal muscle strips of rat colon were placed in an organ bath and subjected to 1 g of basal tension. After 30 min of equilibration, strips were incubated for 15 min in the absence of any agonist or inhibitor (Basal), in the presence of 1  $\mu$ M carbachol (CCh) alone, or in the presence of a selective inhibitor plus 1  $\mu$ M CCh. Separate strips were used to test the effect of the selective Rho kinase inhibitor Y27632 (10  $\mu$ M), the selective ERK1/2 inhibitor PD98059 (10  $\mu$ M), the selective CaMKK inhibitor STO-609 (10  $\mu$ M) and the selective CaMKII inhibitor KN-62 (10  $\mu$ M). Strips were flash frozen in liquid nitrogen and MLCK activity was measured as described in the methods using MLC<sub>20</sub> as substrate and [<sup>32</sup>P]ATP. MLCK activity was measured as cpm and values are expressed as percent of basal activity. Values are means  $\pm$  SEM of 7–13 experiments and each experimental value derived from several strips. CCh significantly increased ( $P < 0.05$  vs basal) MLCK activity in the presence and absence of each inhibitor. The effect of CCh on MLCK activity was not significantly inhibited ( $P > 0.05$  vs CCh) in the presence of any of the inhibitors.

The inhibitory effect of Y27632 in combination with KN-62 was significantly greater than the effect obtained with KN-62 alone. Peak contractions were  $0.92 \pm 0.09$  grams for CCh alone,  $0.60 \pm 0.05$  grams for CCh plus KN-62 ( $P < 0.05$ ), and  $0.36 \pm 0.06$  grams for CCh plus KN-62 and Y27632 ( $P < 0.01$ ) (Fig. 9A). For AUC the values (in grams-seconds) were  $80.97 \pm 11.84$  for CCh alone,  $51.43 \pm 4.00$  for CCh plus KN-62 ( $P < 0.05$ ,  $n=8$ ), and  $34.70 \pm 3.18$  for CCh plus KN-62 and Y27632 ( $P < 0.05$ ,  $n=8$ ) (Fig. 9B). Thus, there is a significant ( $P < 0.05$  for both peak and AUC contraction) additive effect of CaMKII inhibition and Rho Kinase inhibition on both peak contraction and AUC.

##### 5) Effect of kinase inhibitors on MLCK activity

The effect of each kinase inhibitor was examined to determine if it had a direct effect on MLCK activity induced by 1  $\mu$ M CCh. CCh caused a significant increase in MLCK activity ( $65 \pm 18\%$  above basal). There was no significant effect of any of the kinase inhibitors on the MLCK kinase activity induced by 1  $\mu$ M CCh ( $P > 0.05$  vs 1  $\mu$ M CCh alone for each kinase) (Fig. 10).

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## Discussion

Gastrointestinal motility is mediated by the coordinated contractile activity of smooth muscle cells of both circular and longitudinal muscle layer. The main excitatory neurotransmitters in the gastrointestinal tract are acetylcholine and substance P (1–3). This study demonstrated that contraction in longitudinal muscle strips,

both peak contraction and total contraction (measured as AUC), in response to the acetylcholine mimetic, carbachol, was solely dependent on activation of m3 receptors. Similar m3 receptor-dependent contraction was also reported in smooth muscle from circular muscle layer (37). The results are consistent with the concept that m3 receptors are known to play a dominant role in eliciting smooth muscle contraction and m2 receptors are considered to play a minor role despite their abundant expression (45–48, 50). Physiologically *in vivo* activation of the m2 receptor augments smooth muscle contractions mediated by m3 receptors. This is consistent with the concept of the conditional role of the m2 receptors in the smooth muscle (45, 46). Studies by Unno et al. (48), using m2 and m3 receptor knockout mice and pertussis toxin (PTx) to block m2-mediated contractions, have demonstrated that both m2 and m3 receptor activation induces ileal muscle contraction and the contribution of m2 receptors to contraction depends on the concentration of carbachol; at less than 1  $\mu\text{M}$  carbachol, nearly 80% of the contractions are PTx sensitive and at concentrations more than 10  $\mu\text{M}$  carbachol, PTx had no significant effect suggesting that the contribution of m2 receptors to CCh-induced contraction is significant only at low CCh concentrations and decreases with increasing concentrations of CCh. The notion that the effect of CCh in innervated longitudinal muscle strips could be due to activation of neuronal receptors was excluded as blockade of neuronal activation with tetrodotoxin had no effect on CCh-induced peak and total contraction.

Previous studies in isolated muscle cells from circular and longitudinal muscle layer have shown in circular muscle that treatment with CCh induced activation of Rho kinase downstream of RhoA, although the upstream mechanism of RhoA are distinct in circular versus longitudinal muscle cells. M3 receptors are coupled to  $G_{12}$  to activate RhoA via RhoGEF, LARG in longitudinal muscle cells, whereas m3 receptors are coupled to  $G_{13}$  to activate RhoA via RhoGEF, p116RhoGEF in circular muscle cells (37, 43, 44). One of the downstream targets of RhoA is serine/threonine kinase Rho kinase, which plays an important role in the regulation of sustained contraction. *In vivo* studies demonstrated the phosphorylation at Thr696/853 of MYPT1, the regulatory subunit of MLCP, and *in vitro* studies demonstrated phosphorylation at Thr38 of CPI-17, an endogenous inhibitor of MLCP; phosphorylation of both substrates leads to inhibition of MLCP activity and an increase in  $\text{MLC}_{20}$  phosphorylation and muscle contraction (18–20, 51). Inhibition of both basal tone and CCh-induced peak and total contraction by blockade of Rho kinase with Y27632 supports the role of Rho kinase in not only maintenance of tone but also agonist-induced contraction and may reflect stimulation of basal and disinhibition of agonist-induced inhibition of MLCP activity. Studies by Hagerty et al., offers an alternative explanation whereby Rho kinase increases the activity of ZIP kinase, a putative MLC kinase (52). This is supported by Ihara and MacDonald, who demonstrated a direct phosphorylation of  $\text{MLC}_{20}$  by ZIP kinase as well as phosphorylation of MYPT1 by ZIP kinase, both lead to increased contraction (53). A direct phosphorylation of  $\text{MLC}_{20}$  by Rho kinase on  $\text{MLC}_{20}$  has also been demonstrated in *in vitro* studies (24). Regulation of multiple proteins involved in the regulation of MLCP by Rho kinase is also indicative of a stronger inhibitory effect of Y27632 on total contraction than peak contraction. It is noteworthy that as compared to inhibition of other kinases, inhibition of Rho kinase resulted in greater inhibition of peak and total contraction. The effect of Y27632 on high  $\text{K}^+$ -induced smooth muscle contraction was demonstrated in several studies. In rat thoracic aorta and mesenteric artery, inhibition of  $\text{K}^+$ -induced contraction by Y27632 was attributed to disruption of actin filament network, but not to changes in MLCK and MLCP activities (54). In rat caudal artery, Y27632 had no effect on  $\text{K}^+$ -induced increase in  $\text{Ca}^{2+}$ , but abolished sustained contraction (55). In chicken gizzard, a typical phasic muscle, inhibition of  $\text{K}^+$ -induced contraction was not significant even in the presence of 10  $\mu\text{M}$  of Y27632 (56). These results suggest that the effect of Y27632 is variable depending on the species and muscle type. The variable responses of Y-27632 on high  $\text{K}^+$ -induced contraction may be also caused by expression of  $\text{Ca}^{2+}$ -dependent PI3 kinase that mediates RhoA activation (57).

Extracellular signal-regulated kinases (ERK1/2) play an important role in the regulation of smooth muscle contraction via phosphorylation of caldesmon or regulation of kinases such as MLCK or CaMKII (21, 58–60). The involvement of ERK1/2 was shown to be dependent on the type of smooth muscle and agonist. In rat aorta, PD98059 had no effect on  $K^+$ -induced contraction, but inhibited lysophosphatidylcholine-induced augmentation of  $K^+$ -induced contraction (61). In lower esophageal sphincter of rat or cat, PD98059 reduced agonist-induced contraction (62, 63). In swine carotid arteries, PD98059 had no effect on histamine-induced contraction (64), whereas in ferret aorta PD98059 had no effect on  $K^+$ -induced contraction, but significantly inhibited phenylephrine-induced contraction in the absence, but not in the presence of extracellular  $Ca^{2+}$  (59). Activation of ERK1/2 in response to CCh has been demonstrated previously in muscle cells isolated from circular and longitudinal muscle layers; in both cell types activation of ERK1/2 induces stimulation of cytosolic phospholipase  $A_2$  (cPLA<sub>2</sub>) activity, which plays an important role in  $Ca^{2+}$  mobilization mechanisms in longitudinal muscle cells (8, 13, 49, 65).  $Ca^{2+}$  mobilization in these muscle cells is mediated by activation of cPLA<sub>2</sub> and generation of arachidonic acid (AA), leading to  $Ca^{2+}$  influx and stimulation of cyclic ADP ribose (cADPR); both  $Ca^{2+}$  and cADPR act in concert to induce  $Ca^{2+}$  release via ryanodine receptors (RYR2)/ $Ca^{2+}$  channels (9, 13). Inhibition of ERK1/2 activity caused reduction in both peak and total contraction and the degree of inhibition second only to Rho kinase inhibition. Like Rho kinase, ERK1/2 targets multiple proteins to regulate MLC<sub>20</sub> phosphorylation and contraction. Previous studies have shown that activation of integrin-linked kinase, another putative MLC kinase, and MYPT1 kinase is dependent on ERK1/2 (28, 60), suggesting that ERK1/2 can regulate contraction either as MLC kinase or an inhibition of MLCP activity. A direct stimulatory effect of ERK1/2 on MLCK was also demonstrated (22). Inhibition of both Rho kinase and ERK1/2 had no additive effect suggesting that the pathways activated by Rho kinase and ERK1/2 to regulate MLC<sub>20</sub> phosphorylation and contraction may not line in parallel.

Previous studies in muscle cells isolated from circular and longitudinal muscle layers show that MLCK activity in response to muscarinic receptor activation is negatively regulated via phosphorylation of MLCK at Ser815 by AMPK, which is activated downstream of CaMKK $\beta$  upon phosphorylation at Thr<sup>172</sup> (32, 43). STO609, an inhibitor of CaMKK $\beta$  (66), blocked muscarinic receptor-induced phosphorylation of AMPK and MLCK resulting in attenuation of AMPK activity and augmentation of MLCK activity, MLC<sub>20</sub> phosphorylation and muscle contraction in circular muscle. In contrast, in longitudinal strips as shown in the present study, inhibition of CaMKK activity was found to reduce peak and total contraction but had no effect on MLCK activity. The distinct role of CaMKK $\beta$  in the regulation of MLCK activity and muscle contraction in the innervated muscle strips compared to isolated muscle awaits further work.

Inhibition of another  $Ca^{2+}$ /CaM-dependent enzyme CaMKII also reduced CCh-induced peak and total contraction. These results are also in conflict with the previous studies in tracheal smooth muscle demonstrating that CaMKII phosphorylates MLCK and inhibits its activity by decreasing affinity for  $Ca^{2+}$ /CaM (38). Kim et al, however, demonstrated direct phosphorylation of MLC<sub>20</sub> with CaMKII activation in ferret aorta. CaMKII also has positive modulatory effects on  $Ca^{2+}$  channels that may lead to increase in contraction. Another possibility could be inhibition of ERK1/2 activity and ERK1/2-dependent contractile process as CaMKII was shown to be an upstream effector of ERK1/2 (58, 67). Thus blockade of CaMKII would affect contraction in a manner similar to ERK1/2 inhibition.

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## Conflict of Interest

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The authors declare that they have no conflict of interest.

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