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Effect of Lithium on Smooth Muscle Contraction and Phosphorylation of Myosin Light Chain by MLCK

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Summary

The aims of our study were to investigate into the effect of lithium on smooth muscle contraction and phosphorylation of myosin light chain (MLC_{20}) by MLCK and to find out the clue of its mechanism. Isolated rabbit duodenum smooth muscle strips were used to study the effects of lithium on their contractile activity under the condition of Krebs' solution by means of HW-400S constant temperature smooth muscle trough. Myosin and MLCK were purified from the chicken gizzard smooth muscle. Myosin phosphorylation was determined by Glycerol-PAGE, myosin Mg^{2+}-ATPase activity was measured by Pi liberation method. Lithium (10-40 mM) inhibited the contraction in duodenum in a dose-related and time-dependent manner. Lithium could also inhibit the extent of myosin phosphorylation in a dose-related and time-dependent manner, whereas it inhibited Mg^{2+}-ATPase activity in a dose-related manner. Lithium inhibited smooth muscle contraction by inhibition of myosin phosphorylation and Mg^{2+}-ATPase activity.

Key words

Lithium • Smooth muscle contraction • Phosphorylation of myosin light chain (MLC_{20}) • Myosin Mg^{2+}-ATPase

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Introduction

The pharmacological actions of lithium are widespread. It can be a useful medication in the treatment of aggressive behavior and affective instability. It is not only able to reverse neurological damage induced by *Vinca* alkaloids, to act on myoneural preparations of rats (Abdel-Zaher 2000) and to improve the behavioral disorder in rats subjected to transient global cerebral ischemia, but it can also regulate hippocampal neurogenesis by ERK pathway and facilitate recovery of spatial learning and memory in rats after transient global cerebral ischemia (Yan *et al.* 2007). Thus it can effects various neuroprotective effects.

In higher concentrations (10 mM) lithium inhibits *in vitro* functions of human polymorphonuclear leukocytes (PMNL) and lymphocytes (Anderson *et al.* 1982). On the other hand, it is related to diabetic disease (Hu *et al.* 1999) and its gastrointestinal complications is the most common one (Ozturk *et al.* 1992). Thus, its mechanism is perhaps related to glycogenic pathway (Kashour *et al.* 2003).

Moreover, lithium has many toxic or side effects. It has significant potential for neurotoxicity in normal population (Gill *et al.* 2003) and hyperthyroidism (Sadoul *et al.* 1994). It also disturbs the water-electrolyte equilibrium and affects the level of essential elements (Kielczykowska *et al.* 2003). A significant number of babies, which were exposed to lithium during gestation, suffered from developmental neuron deficits and depressed neurological status (Kozma 2005).

Therefore, it is widespread and highly effective drug in clinical practice (Ide *et al.* 2009). However, there are also many side effects (Diekmann 2000), particularly gastrointestinal ones including nausea, vomiting, diarrhea, anorexia, abdominal pain and dry mouth.

Though it was reported that lithium could act on endothelial-dependent relaxation in isolated rat aorta and on angiotensin II-stimulated vascular contraction

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(Dehpour *et al.* 1995), but the meachanism of its action is still not fully understood.

We are interested in the effects of lithium on muscle contractile function, because early symptoms of toxic cases have a relationship to gastrointestinal complications (Ozturk *et al.* 1992, Diekmann 2000). By investigating this mechanism we hope to find the way how to reduce these side effects of lithium.

Some believe that lithium exerts its pharmacological action by inhibiting the release of N-acetyl-β-glucosaminidase induced by neomycin in the rat heart (Dehpour *et al.* 1995), by inhibiting substance P- and vasoactive intestinal peptide-induced relaxations on isolated porcine ophthalmic artery (Vincent 1992) or by influencing membrane adenosine triphosphatases in certain postural muscles of rats (Adipudi and Reddy 1994).

Therefore, we propose to study whether lithium can influence the smooth muscle contraction in isolated duodenum and the phosphorylation of myosin light chain (MLC_{20}) by MLCK level at the same time. The phosphorylation of MLC_{20} by MLCK is generally considered as the main primary mechanism for the regulation of the smooth muscle contraction. Phosphorylation can be simply described as an interaction of Ca^{2+} with CaM that induces a conformational change and activation of MLCK. The activated MLCK catalyzes phosphorylation of MLC_{20}. The phosphorylation of MLC_{20} triggers cycling of myosin cross-bridges along actin filaments and the development of force (Tang *et al.* 2005).

Methods and Materials

Animals

The experiments were performed according to the rules of animal care and were approved by the Animal Protection Committee of Dalian Medical University. Rabbits (New Zealand, 1.5-2 kg) were used in all experiments. Animals were housed five per cage in a temperature-controlled room with a 12-h light-dark cycle (lights on at 7:00 a.m.). Food and water were available *ad libitum*.

Chemicals and instruments

Lithium carbonate was purchased from Sigma-Aldrich (grade SigmaUltra; CAS Number: 554-13-2), Krebs solution contained (in mM) sodium chloride 114.0, potassium chloride 4.7, magnesium chloride 1.2, calcium chloride 2.5, sodium dehydrogenate phosphate 1.8, glucose 11.5, sodium bicarbonate 18.0, pH 7.4±0.5.

HW-400S constant temperature smooth muscle trough and BL-420F biology function experiment system were from Chengdu Tai Meng Science and Technology Ltd Co.

Perfusion of isolated duodenal segments and gastrointestinal motility

The abdomen was opened under urethane anesthesia. The duodenum between the pylorus and the Treitz ligament was removed and luminally perfused *ex vivo* as described earlier (Schümann and Hunder 1996). Briefly, the perfusion conditions were as follows: recirculation luminal perfusion with 30 ml of bicarbonate-buffered Tyrode solution (37 °C, pH 7.2), which was equilibrated with 95 % O_2, 5 % CO_2, 25 cm H_2O hydrostatic pressure, and 50 ml/min flow rate. The segments were kept in a moist chamber.

Contractions of the proximal duodenum were directly monitored by both frequency (contractions per minute) and amplitude (increase in pressure). In some cases, a motility index (frequency times mean amplitude) was calculated. Occasional movement artifacts were easily identified as spikes that appeared simultaneously in both recorded channels, and they were eliminated from data analysis. To determine the time at which tolerance developed to the motility effects of lithium, we compared the frequency of contractions in control recordings with the frequency of contractions after lithium infusion in different groups of animals, each animal served as its own control, and recording of intestinal motility was limited to 6 h.

Drug administration

Lithium carbonate was administered according to previous articles (Anderson *et al.* 1982, Ravichandran *et al.* 1998, Erdal *et al.* 2005). Its pH was adjusted to 7.4 before it was used. It was injected into experimental chamber with mini-syringe. The dose for continuous administration of lithium was chosen to establish a steady-state concentration of lithium in the isolated intestine approximately equal to the D_{50}, the effective dose of lithium that results in 50 % increasing of transit.

Continuous administration of lithium (5, 10, 20, 40 mM, respectively) decreased the frequency and amplitude of contractions in the duodenum. To determine the time at which a tolerance developed to the motility effects of lithium, we compared the frequency and amplitude of contractions in control recordings with the

frequency and amplitude of contractions at various times after the initiation of lithium infusion. Lithium infusion resulted in a significant decrease of contractility in the duodenum within 1 h and its effects persisted for 12-15 h. Tolerance of the contractile activity of the intestine to continuous administration of lithium was assessed either by measuring the time required for the duodenum to return to normal frequency of contractions or by recording the loss of effectiveness of lithium administration, which suggested that the intestinal contractile activity was tolerant to the effects of continuous lithium administration (5, 10, 20, 40 mM, respectively).

Protein purification

The myosin and MLCK used in our study were purified from the chicken gizzard smooth muscle to homogeneity as we described previously (Tang *et al.* 2005). The purity analysis of protein samples was assessed by SDS-PAGE.

MLC_{20} phosphorylation

MLC_{20} phosphorylation was carried out in 20 mM Tris-HCl (pH 7.4) buffer containing 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 60 mM KCl, 2 mM ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 4 μM myosin and 2 mM ATP. Various concentrations of MLCK, in different incubation time and incubation temperature for MLC_{20} phosphorylation are described in detail in the corresponding figure legends.

Determination of MLC_{20} phosphorylation

Glycerol-polyacrylamide gel electrophoresis (Glycerol-PAGE) was used to measure the extent of MLC_{20} phosphorylation. Glycerol-PAGE was made using the separating gel containing 13.96 % acrylamide, 0.372 % bis-acrylamide, 40 % (v/v) glycerol, and 0.375 M Tris(pH 8.7); and the stacking gel containing 5.72 % acrylamide, 0.152 % bisacrylamide, 10 % (v/v) glycerol and 0.125 M Tris-HC1 (pH 6.7). Myosin samples which contained 7.5 M urea were added to the sample buffer containing 6 M urea, 20 % glycerol, 0.05 M Tris (pH 6.7), 14 mM β-ME, and a moderate amount of 0.01 % Bromophenol Blue (BPB). The reaction mixture was loaded onto the gel.

The densitometry extent of MLC_{20} phosphorylation was measured by Scion Image software (Scion Co. Ltd). The extent of diphosphorylation (DIP) of MLC_{20} was selected as the control (calculated as 100 %); the extent of monophosphorylation (MIP) of MLC_{20} was a relative value calculated from MIP/DIP.

Myosin Mg^{2+}-ATPase activity measurement

The method for measuring Mg^{2+}-ATPase activity of myosin was described previously (Lin 2000).

Other procedures

Protein concentrations were determined by the method of Bradford (1976).The graphs of phosphorylation of MLC_{20} and myosin Mg^{2+}-ATPase activities were obtained with Microsoft Excel 2005.

Statistical analysis

The results are expressed as means ± S.D. Statistical analysis was performed with unpaired t test in the organ chamber experiment, and with one-way ANOVA in the other experiments. $P<0.05$ was considered to indicate a statistical difference.

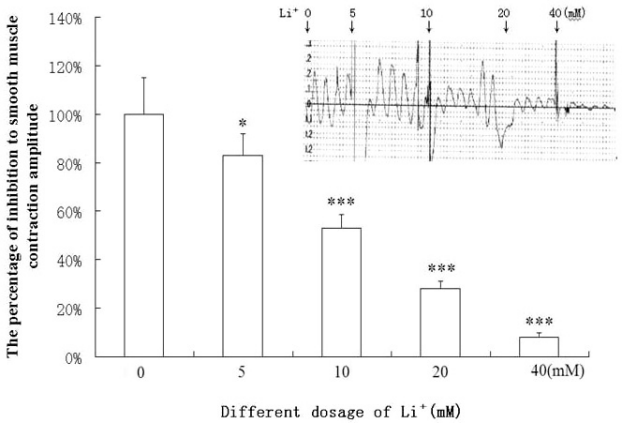

Fig. 1. Effect of lithium on the amplitude of smooth muscle contraction in different lithium concentrations. The inset represents the diagram of scope and frequency curves of smooth muscle contraction. The arrows show that the contraction scope and frequency were changed at different lithium concentrations. Asterisks indicate significant differences between particular lithium concentration groups (*** $P<0.001$, * $P<0.05$). Data are means ± S.D. (n=8).

Results

Effect of different lithium concentration on the amplitude of smooth muscle myosin contraction

The dose-response curve for lithium-induced decrease of rabbit small duodenum contraction is shown in Figure 1. The D_{50} was calculated from the linear portion of the dose-response curve (20-80 % of maximum), using a linear regression analysis. The administration of lithium (5, 10, 20, 40 mM, respectively)

resulted in an inhibition of contraction amplitude in duodenum (from 100±11.2 % to 85±0.89 %, 51±0.54 %, 25±0.31 %, and 5.0±0.52 %) (Fig. 1) in a dose-dependent manner. There was a significant difference between the amplitude of group (5 mM) and the corresponding negative control (P<0.05). This difference became more significant at higher lithium concentrations (P<0.001).

Effect of lithium on the amplitude of smooth muscle myosin contraction at different incubation times

The administration of lithium (10 mM) for 4, 8, 12, 16 and 20 min, respectively, resulted in an inhibition of the amplitude of duodenum contractions (from 100±10.2 % to 85±0.86 %, 64±0.59 %, 43±0.51 %, 24±0.27 %, and 8.0±0.84 %) (Fig. 2) in a dose-dependent manner. There was a significant difference between the amplitude of group (4 min) and the corresponding negative control (P<0.05). This difference was more significant at prolonged incubation time (P<0.001).

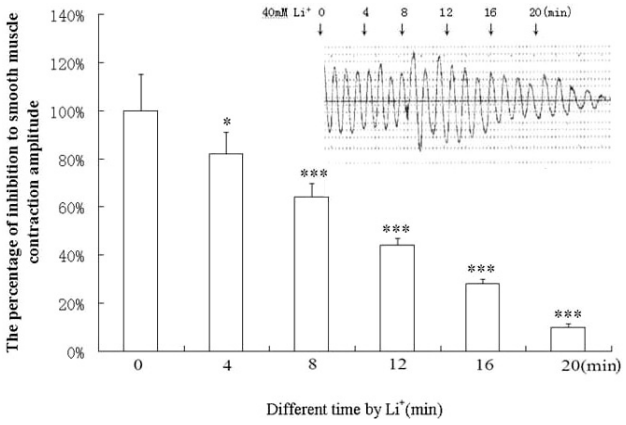

Fig. 2. Effect of lithium on the amplitude of smooth muscle contraction at different incubation time. The inset represents the diagram of scope and frequency curves of smooth muscle contraction. The arrows show that the contraction scope and frequency were changed under the 20 mM lithium concentration at different incubation times. Asterisks indicate significant differences between particular incubation time groups (*** P<0.001, * P<0.05). Data are means ± S.D. (n=8).

Effect of different lithium concentrations on MLC_{20} phosphorylation by MLCK

The high sensitivity and efficacy of MLC_{20} phosphorylation by MLCK in the presence of Ca^{2+}, CaM and low concentration of MLCK is well known (Tansey *et al.* 1994). Thus myosin (1 μM) could be phosphorylated with at least 0.005 μM MLCK. We have therefore chosen 2 μM MLCK and 4 μM myosin concentrations for MLC_{20} phosphorylation. The lanes 0-4 (Fig. 3), which depict negative control (without MLCK and lithium), positive control (without lithium) as well as 5, 10 and 20 mM lithium, indicate 0 %, 100±9.91 %, 87±0.85 %, 50±0.53 %, and 34±0.36 % of MLC_{20} phosphorylation. The extent of phosphorylation in lanes 2, 3 and 4 was decreasing with increased lithium concentration in a dose-dependent manner. There was a significant difference between the amplitude of group (5 mM) and the corresponding positive control (0 mM) (P<0.05). This difference became more significant at higher lithium concentrations (P<0.001).

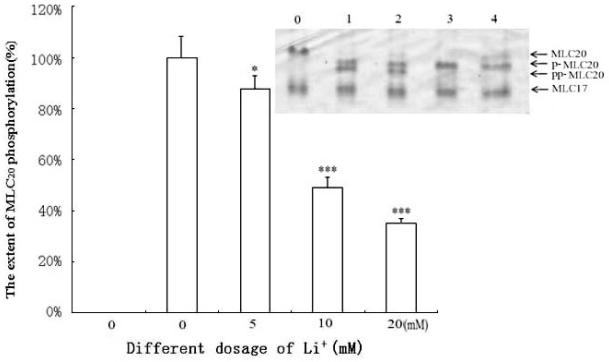

Fig. 3. Effect of lithium on MLC_{20} phosphorylation by MLCK at different lithium concentrations. The lanes 0-4 show the negative control (blank control without incubation), positive control (without lithium), 5, 10, 20 mM lithium concentration group in the same incubation time. 4 μM myosin (1.76 $mg \cdot ml^{-1}$) were used at 25 °C for 20 min in the incubation. Gizzard smooth muscle myosin, the 20,000 Mr light chain (L_{20}) of which is phosphorylated in vitro with a calmodulin-myosin light chain kinase system. It is separated into 5 isolated bands on a Glycerol-PAGE. Their mobilities were in the following order: myosin with unphosphorylated L_{20} (L_{20}) < monophosphorylatedL_{20} (p-L_{20}) < myosin with diphosphorylated L_{20} (pp-L_{20}) < myosin with unphosphorylated L_{17} (L_{17}). MLC_{20} represents unphosphorylated 20 KDa myosin light chain; p-MLC_{20} represents monophosphorylated 20 KDa myosin light chain; pp-MLC_{20} represents diphosphorylated 20 KDa myosin regulatory light chains; MLC_{17} represents 17 KDa myosin essential light chains. The abscissa was different lithium concentration; the ordinate was the relative extent of MLC_{20} phosphorylation (*** P<0.001, or * P<0.05). Data are means ± S.D. (n=8).

Effect of lithium on MLC_{20} phosphorylation by MLCK at different incubation times

Furthermore, we investigated MLC_{20} phosphorylation by MLCK in the presence of 20 mM lithium for three different incubation times, i.e. 5, 10 and 20 min. The lanes 0-4 (Fig. 4) show 0 %, 97±0.99 %, 81±0.87 %, 50±5.4 %, 27±2.9 % of MLC_{20} phosphorylation. The extent of MLC_{20} phosphorylation in lanes 2, 3 and 4 was decreasing with increasing incubation time in a time-dependent manner. There was a significant difference between the amplitude of group (5 min) and the corresponding positive control (P<0.05) and these

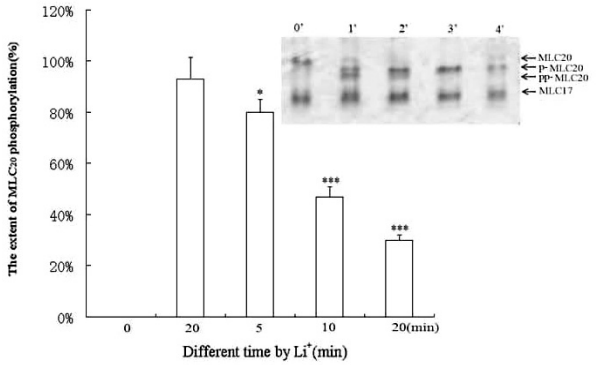

Fig. 4. Effect of lithium on MLC_{20} phosphorylation by MLCK at different incubation times. The lanes 0-4 show the negative control (blank control without incubation), positive control (without lithium), 5, 10, 20 min incubation time group in the 20 mM lithium concentration, respectively. Incubation times for lane 0′ = 0 min, lane 1′ = 20 min (without lithium), lane 2′ = 5 min (with 20 mM lithium), lane 3′ = 10 min (with 20 mM lithium), lane 4′ = 20 min (with 20 mM lithium) were chosen. 4 μM myosin (1.76 $mg \cdot ml^{-1}$) were used at 25 °C. MLC_{20} represents unphosphorylated 20 KDa myosin light chain; p-MLC_{20} represents monophosphorylated 20 KDa myosin light chain; pp-MLC_{20} represents diphosphorylated 20 KDa myosin regulatory light chains; MLC_{17} represents 17 KDa myosin essential light chains. Significant differences: *** P<0.001, * P<0.05 compared to the corresponding controls. Data are means ± S.D. (n=8).

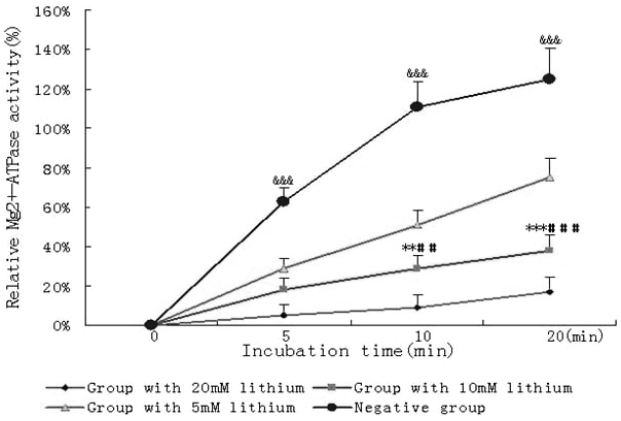

Fig. 5. Effect of different lithium concentrations on myosin Mg^{2+}-ATPase activities at different incubation times. The curve ◆, ■, ▲ and ● represents the Mg^{2+}-ATPase activities in groups with 20 mM, 10 mM, 5 mM lithium and negative group respectively. 4 μM myosin and 2 μM MLCK were used in the assay for different incubation times, i.e., 5, 10, 20 min. Significant differences: &&& P<0.001 compared with other groups with 5, 10, 20 mM lithium; ** P<0.01 compared. Data are means ± S.D. (n=8).

differences became more significant at increasing incubation time (P<0.001).

Effect of different lithium concentration on myosin Mg^{2+}-ATPase activities at different incubation times

Figure 5 shows that myosin Mg^{2+}-ATPase activities measured at three lithium concentrations were all enhanced with prolonging of the incubation time. Our results indicated that at all incubation times, the highest Mg^{2+}-ATPase activity was observed when myosin was studied in the negative group, and the enzyme activity decreased progressively with increasing lithium concentration. The differences between Mg^{2+}-ATPase activities in different groups become more obvious with the extension of incubation time. There were significant differences between each lithium group and negative group (P<0.001) and between incubations for 10 and 20 min (P<0.01).

Discussion

Our results (Figs 1 and 2) indicated that lithium (10-40 mM) could inhibit the contraction in duodenum in a dose-related and time-dependent manner. It is most likely caused by the inhibition of myosin phosphorylation and Mg^{2+}-ATPase activity (Figs 3-5).

There are controversies on the pharmacological actions of lithium and their mechanisms. People general thought that lithium enhances locomotor activity induced by DOI and Fos-like immunoreactivity (Moorman and Leslie 1998). On the other hand, lithium can downregulate PKB/Akt and cyclin E in hepatocellular carcinoma cells (Erdal *et al.* 2005). Lithium also regulates PKC-mediated intracellular cross-talk and gene expression in the CNS *in vivo* (Chen *et al.* 2000) and inactivates Gi modulation of adenylate cyclase in brain (Monica *et al.* 1992). Though lithium could inhibit glycogen synthase kinase-3, it could not pose a higher risk for the development of cancers of the Wnt pathway (Gould *et al.* 2003). In addition, lithium regulates the proliferation of stem-like cells in retinoblastoma cell lines possibly through the canonical Wnt signaling pathway (Silva *et al.* 2010). It has also been observed that lithium carbonate can interact with L-tryptophan (Song Le *et al.* 2009).

However, there are also different opinions on the mechanism of gastrointestinal response. It was reported that lithium (45 mM) inhibits the Na^+/Ca^{2+} exchanger (Kupriyanov *et al.* 1997) and activates mammalian Na^+/H^+ exchanger in muscle (Kobayashi *et al.* 2000). Thus it can diminish Ca^{2+} entry through Na^+-Ca^{2+} exchanger. Moreover, lithium decreases all mechanical and energetic properties of paired-pulse contractions (Savio-Galimberti and Ponce-Hornos 2006). However, other investigators believe that lithium (45 mM) decreases pressure developed and pressure-time integral,

respectively. Lithium possibly acts on an additional myocardial Ca^{2+} sensitive locus (different from the Na^{+}-Ca^{2+} exchanger) (Bonazzola *et al.* 2002). However, lithium in a high concentration decreases significantly Ca^{2+} mobilization only in the presence of ML-9. These results suggest that the mechanism of lithium action may include a compensatory effect on MLCK modulation (Suzuki *et al.* 2004).

We used Glycerol-PAGE to analyze the phosphorylated state of gizzard smooth muscle. Thus we could evaluate the activity of MLCK by observing the mono- or di-phosphorylated myosin on Glycerol-PAGE (Takano-Ohmuro and Kohama 1986, Tang *et al.* 2005). It is known that phosphorylation of myosin regulatory light chain (RLC) at Ser19 (monophosphorylation) promotes filament assembly and enhances actin-activated ATPase activity, while phosphorylation at both Ser19 and Thr18 (diphosphorylation) further enhances the ATPase activity (Uehara *et al.* 2008). Our results confirmed that lithium (10-45 mM) inhibited the contraction of intestinal muscle in a dose-related manner and in a time dependent manner. Similarly, lithium also inhibits the extent of myosin phosphorylation in a dose-related and time-dependent manner.

Our experiments indicated that the results about phosphorylation of MLC_{20} were paralleled with those about Mg^{2+}-ATPase activity. Lithium also inhibited the extent of Mg^{2+}-ATPase activity in a dose-related manner. Nevertheless, the Mg^{2+}-ATPase can catalyses the hydrolysis of myosin ATP in the presence of actin or calcium ions to form myosin ADP and orthophosphate. This reaction is the immediate source of free energy that drives muscle contraction. Hydrolysis of ATP by myosin is directly related to contractile function and structural changes in the head region of myosin during a contraction (Thomas *et al.* 1995). It is likely that the changes in myosin enzymatic activity accompany the changes in myosin structure and function. For this reason, the lower was Mg^{2+}-ATPase activity, the lower muscle contraction force was developed.

Our results could explain the molecular mechanism of gastrointestinal side effects, i.e. the effect of lithium on the MLC_{20} phosphorylation by MLCK. Lithium inhibited smooth muscle contraction through the inhibition of myosin phosphorylation and Mg^{2+}-ATPase activity. In the future we shall study how lithium interacts with the effective region of MLCK.

Conflict of Interest

There is no conflict of interest.

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