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Effect of lithium on smooth muscle contraction and phosphorylation of myosin light chain by MLCK

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Abstract

OBJECTIVE To investigate into the effect of lithium on smooth muscle contraction and phosphorylation of myosin light chain (MLC_{20}) by MLCK. Try to find out the clue of its mechanism. **METHODS** An isolated rabbit duodenum smooth muscle strip was selected in an assay to study the effects of lithium on their contractile activity under the condition of Krebs' solution using HW-400S constant temperature smooth muscle trough. Myosin and MLCK used in our study 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. **RESULT** Lithium (10-45mM) inhibited the contraction in duodenum in a dose-related manner and in a time dependent manner; Lithium also could inhibit the extent of phosphorylation of myosin in a dose-related manner and in a time dependent manner; and lithium inhibited the extent of Mg^{2+}-ATPase activity in dose-related manner. C**ONCLUSION** Lithium inhabited smooth muscle contraction by the way of inhibition of myosin phosphorylation and Mg^{2+}-ATPase activity.

Key word: lithium, smooth muscle contraction, phosphorylation of myosin light chain (MLC_{20}), myosin Mg^{2+}-ATPase

Introduction

The pharmacological actions of lithium are widespread. It can be a useful medication in the treatment of aggressive behavior, affective instability. Not only it is able to reverse neurological damage induced by vinca alkaloids and act on myoneural preparations of rats (ABDEL-ZAHER AO 2000) and even improve the behavioral disorder in rats subjected to transient global cerebral ischemia，but also it can regulate hippocampal neurogenesis by ERK pathway and facilitate recovery of spatial learning and memory in rats after transient global cerebral ischemia (YAN X B at al.

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2007).So it can play various neuroprotective roles.

It is in higher concentrations (10mM) to the inhibition of human polymorphonuclear leukocyte (PMNL) and lymphocyte functions in vitro by lithium (ANDERSON R at al. 1982). On the other side, It is related to diabetic disease (HU M at al. 1999) and its gastro-intestinal complications is the most common one (OZTURK Y at al. 1992). Thus, its mechanism perhaps is related to glycogenic pathway (KASHOUR T at al. 2003).

Moreover, there are many poisonous or side effects to it. It has significant potential for neurotoxicity in population (GILL J at al. 2003) and hyperthyroidism (SADOUL JL at al. 1994), disturbing the water-electrolyte equilibrium and affect the level of essential elements (KIELCZYKOWSKA M at al. 2003). A significant number of babies which exposed to lithium during gestation suffered from neuron developmental deficits and depressed neurological status (KOZMA C 2005).

Therefore, it is widespread and high effective drug in clinical practice (IDE N at al. 2009). However, there are also many side effects (Diekmann U 2000), particularly gastro-intestinal tract response. Gastrointestinal side effects including nausea, vomiting, diarrhea, anorexia, abdominal pain, and dry mouth have been reported frequently

Though it reported that lithium could act on endothelial-dependent relaxation in rat isolated aorta and on angiotensin II-stimulated vascular contraction so on (DEHPOUR AR at al. 1995).

But still it was not clear for people to understand the mechanism of its accurate function.

What we are interested in is about the effects of lithium on muscle contractile function, because early symptoms of toxic cases have a relationship with gastro-intestinal complications(OZTURK Y at al. 1992, Diekmann U 2000). What we will do could provide a foundation to find the way to reduce side effect through investigating its mechanism.

Some believe that lithium play the pharmacological action by inhibiting the release of N-acetyl-beta-glucosaminidase induced by neomycin in the rat heart (DEHPOUR AR at al. 1995) or by inhibiting substance P and vasoactive intestinal peptide-induced relaxations on isolated porcine ophthalmic artery (VINCENT MB 1992). Or perhaps by influencing membrane adenosine triphosphatases in certain postural muscles of rats (ADIPUDI V and REDDY VK 1994).

Therefore, we propose that whether lithium is related to on the smooth muscle contraction in isolated organ and phosphorylation of myosin light chain (MLC_{20}) by MLCK level in the same time.

Because the main mechanism of smooth muscle contraction is: phosphorylation of MLC_{20} by MLCK is generally considered as the primary mechanism for regulating the contraction of smooth muscle. Phosphorylation can be simply described as an interaction of Ca^{2+} with CaM that induces a conformational change of MLCK and activates 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 Z at al. 2005). Study to this aspect is as follows.

Methods and materials

1.1 Animals, Agents and Instrument

The experiments were performed according to the rules of animal care and were approved by the local animal protection committee (The animal protection committee of Dalian medical university).

Rabbits (New Zealand, 1500-2000g) were used in all experiments. Animals were housed five per cage in a temperature-controlled room with a 12-hr light-dark cycle (lights on at 7:00A.M.). Food and water were available.

Agent: Lithium carbonate: Sigma-Aldrich; grade SigmaUltra; CAS Number: 554-13-2.

Krebs's solution ingredient (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 , BL-420F biology function experiment system, Chengdu Tai Meng Science and Technology Ltd Co.

1.2 Preparation, 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 K., and G. HUNDER 1996). Briefly, the perfusion conditions were as follows: recirculation luminal perfusion with 30 ml of bicarbonate-buffered Tyrode solution (37°C, pH7.2), which was equilibrated with 95% O_2, 5% CO_2, 25cm 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 hr.

Drug administration: The final concentration of lithium was administered according to the articles (ANDERSON R at al. 1982, ERDAL E at al. 2005, RAVICHANDRAN D at al. 1998). 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 intestinal organ 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, 40mM respectably) decreased the frequency and amplitude of contractions in the duodenum. To determine the time at which 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 initiation of lithium infusion. Lithium infusion resulted in a significant decreasing of contractility in the duodenum within 1 hr, and its effects persisted for 12 to 15 hr. Tolerance to continuous administration of lithium in the contractile activity of the intestine was assessed by two means, i.e., by measuring the time required for the duodenum to return to normal frequency of contractions and by recording the loss of effectiveness of administration of lithium (5, 10, 20, 40mM respectably),which suggested that the contractile activity of the intestine was tolerant to the effects of continuous lithium administration (5, 10, 20, 40mM respectably).

1.3 Protein Purification

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

1.4 MLC_{20} Phosphorylation

MLC_{20} phosphorylation was carried out in a 20mM Tris-HCl (pH 7.4) buffer containing 1mM dithiothreitol (DTT), 5mM $MgCl_2$, 60mM KCl, 2mM ethylene glycol bis (beta-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 4μM myosin and 2mM 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.

1.5 Determination of MLC_{20} Phosphorylation and Measurement of MLC_{20} Phosphorylation

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

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

1.6 Myosin Mg^{2+}-ATPase Activity Measurement

The method for measuring Mg^{2+}-ATPase activity of myosin is as described previously (LIN Y 2000).

1.7 Statistical Analysis

The results are expressed as means ±standard deviation ($\bar{x}$±s). 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.

1.8 Other Procedures

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

Results

2.1 Effect of lithium on the amplitude of smooth muscle myosin in different Lithium concentration

The dose-response curve for lithium-induced decreasing of small duodenum in rabbits is shown in Fig.1. The D_{50} was calculated from the linear portion of the dose-response curve (20-80% of maximum), using a linear regression analysis. In Fig.1, Lithium administration of lithium (5, 10, 20, 40mM respectably) resulted in an inhibition of the amplitude of contractions (from 100±11.2% to 85±0.89%, 51±0.54%, 25±0.31% 5.0±0.52% gradually for inhibition percentage, n = 8) in duodenum in a dose dependent manner. It was significant difference between the amplitude of group (5mM) and the corresponding negative control (* = $P < 0.05$). It was difference more significantly among the amplitude of group (10mM) , group (20mM) , group (40mM) and the corresponding negative control

(*** = $P < 0.001$).

Fig.1

2.2 Effect of lithium on the amplitude of smooth muscle myosin in different incubation time

In Fig.2, administration of lithium (10mM) for 4, 8, 12, 16, 20min respectably resulted in an inhibition of the amplitude of contractions (from 100±10.2% to 85±0.86%, 64±0.59%, 43±0.51%, 24±0.27%, 8.0±0.84% gradually for inhibition percentage, n = 8) in duodenum in a dose dependent manner. It was significant difference between the amplitude of group (4min) and the corresponding negative control (* = $P < 0.05$). It was difference more significantly among the amplitude of group (8mM) , group (12mM) , group (16mM) , group (20mM)and the corresponding negative control (*** = $P < 0.001$).

Fig.2

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

In Fig.3, The high sensitivity and efficacy of MLC_{20} phosphorylation by MLCK are known as those in the presence of Ca^{2+} and CaM and low concentration of MLCK is needed to phosphorylate MLC_{20} (TANSEY MG at al. 1994). For instance, Myosin (1μM) could be phosphorylated with (at least 0.005μM) MLCK. So we choose 2μM MLCK and 4μM myosin concentrations for MLC_{20} phosphorylation. The lane 0-4 in Fig.1 shows respectively that negative control (without MLCK and lithium), positive control (without lithium), 5, 10, 20mM lithium. Lane 0-4 shows us that it was 0%, 100±9.91%, 87±0.85%, 50±0.53%, 34±0.36% respectively to the extent of myosin phosphorylation (MLC_{20} phosphorylation) (As the Fig.3 Excel showing). i.e., the extent of phosphorylation in lane 2, 3, 4 were decreased along with lithium concentration being increased gradually in a dose dependent manner. It was significant difference between the amplitude of group (5mM) and the corresponding positive control (0mM) (* = $P < 0.05$). It was difference more significantly among the amplitude of group (10mM) , group (20mM) , group (40mM) and the corresponding positive control (0mM) (*** = $P < 0.001$).

Fig.3

2.4 Effect of lithium on MLC_{20} phosphorylation by MLCK in different incubation time

In Fig4, to investigate MLC_{20} phosphorylation by MLCK under the condition of presence with lithium for different incubation time, three different incubation times, i.e., 5, 10, 20min respectably were selected by 20mM lithium.

In Fig.4 lane 0-4 shows us that 0%, 97±0.99%, 81±0.87%, 50±5.4%, 27±2.9% to the extent of MLC_{20} phosphorylation (As the Fig.3 Excel showing). i.e., the extent of MLC_{20} phosphorylation in lane 2, 3, 4 were decreased along with incubation time being increased gradually, in a time dependent manner. It was significant difference between the amplitude of group (5min) and the corresponding positive control (* = $P < 0.05$). It was difference more significantly among the amplitude of group (10min) , group (20mM)and the corresponding positive control (*** = $P < 0.001$).

Fig.4

2.5 Effect of lithium on myosin Mg^{2+}-ATPase activities by 20mM, 10mM, 5mM lithium and negative group in different incubation time respectively

The results in Fig.5 indicated that different incubation time, i.e., 0, 5, 10, 20min respectably were chosen to examine the myosin Mg^{2+}-ATPase activities. It showed that the Mg^{2+}-ATPase activities of the three conditions were all enhanced with prolonging the incubation time. The results indicated that at different incubation time, the highest Mg^{2+}-ATPase activity was observed when myosin was in the negative group, the second one was the group with 5mM lithium; the third one was the group with 10mM lithium and the lowest was the group with 20mM lithium. The difference between the Mg^{2+}-ATPase activities of different groups become more and more obviously along with the extension of incubation time. It was significant between each lithium group and negative group (&&& $P < 0.001$). The difference was significant between incubation time for 10 and for 20min (**$P < 0.01$ or ### $P < 0.001$).

Fig.5

Discussion

It is the fact that there are controversies to the lithium's pharmacological actions and their mechanisms. People general thought that lithium enhance locomotor activity induced by DOI and Fos-like immunoreactivity (MOORMAN JM and LESLIE RA 1998). On the other hand, Lithium can downregulate PKB/Akt and cyclin E in hepatocellular carcinoma cells (ERDAL E at al. 2005). Some people also thought that lithium regulate PKC-mediated intracellular cross-talk and gene expression in the CNS in vivo (CHEN G at al. 2000). Or it inactivates Gi Modulation of Adenylate Cyclase in Brain (MONICA I at 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 TD at al.2003). The others believe that lithium regulates the proliferation of stem-like cells in retinoblastoma cell lines by a potential role for the canonical Wnt signaling pathway (SILVA AK at al. 2010). As to concrete mechanism, someone had observed that how lithium carbonate can interact with L-Trp using an electrospray ionization mass spectrum (SONG LE X at al. 2009).

However, there are the disputed opinions to the mechanism of gastro-intestinal response. People hold that it is possibly related to that lithium (45mM) inhibit the Na^+/Ca^{2+} exchanger (KUPRIYANOV VV at al. 1997) and activates mammalian Na^+/H^+ exchanger in muscle (KOBAYASHI Y at al. 2000). So it can diminish Ca^{2+} entry through Na^+-Ca^{2+} exchanger. Moreover, decrease all mechanical and energetic properties of paired-pulse contractions (SAVIO-GALIMBERTI E and PONCE-HORNOS JE 2006). However, other ones believed that lithium (45mM) decrease pressure developed and pressure-time integral, respectively. Lithium possibly act on an additional Ca^{2+} sensitive locus (different than the Na^+-Ca^{2+} exchanger) (BONAZZOLA P at al. 2002).

Yet, other people argue that lithium in a high concentration decreased significantly Ca^{2+} mobilization only in the presence of ML-9. These results suggest that the mechanism of action of lithium may include a compensatory effect on MLCK modulation (SUZUKI K at al. 2004).

From Fig.3, Fig.4. We used the Glycerol PAGE to analyze the phosphorylated state of Gizzard

smooth muscle. Thus it could be evaluated the activity of myosin by MLCK via the way of observing the mono-phosphorylated or di-phosphorylated myosin on Glycerol PAGE (HIROMI TAKANO-OHMURO, and KAZUHIRO KOHAMA 1986, TANG Z et al. 2005).It is known that phosphorylation of myosin regulatory light chain (RLC) at Ser19 (mono-phosphorylation) promotes filament assembly and enhances actin-activated ATPase activity, while phosphorylation at both Ser19 and Thr18 (di-phosphorylation) further enhances the ATPase activity (RYOTA UEHARA et al. 2008).Our results confirmed that lithium (10-45mM) inhibited the contraction in intestinal muscle in a dose-related manner and in a time dependent manner; Lithium could also inhibit the extent of phosphorylation of myosin in a dose-related manner and in a time dependent manner.

Our experiment about Mg^{2+}-ATPase activity proved further that the result about phosphorylation of MLC_2 was paralleled with the one about Mg^{2+}-ATPase activity. Lithium also inhibited the extent of Mg^{2+}-ATPase activity in 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. Hydrolyzing ATP by myosin is directly related to contractile function and structural changes in the head region of myosin during a contraction (THOMAS DD at al. 1995). It is likely that changes in myosin's enzymatic activity accompany changes in myosin's structure and function. For this reason, in general condition, the lower Mg^{2+}-ATPase activity is, the lower muscle contraction force is. Therefore it was consistent to our results in different research level. i.e., there was no contradictory phenomenon.

Our results could explain well that the molecular mechanism of gastro-intestinal tract side effects, i.e., the target spot by lithium was on the MLC_{20} phosphorylation by MLCK. Lithium inhibited smooth muscle contraction by the way of inhibition of myosin phosphorylation and Mg^{2+}-ATPase activity.

From now on, it need further to be studied about how lithium interacts with the effective region of MLCK because our result is only preliminary study.

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Figure Legends

Fig.1 Effect of lithium on the amplitude of smooth muscle contraction in different lithium concentration

The top right-hand corner part is the diagram of scope and frequency curves of smooth muscle contraction. The arrow showed that the contraction scope and frequency were changed with the difference of 0, 5, 10, 20, 40mM lithium respectively. The excel picture of left-hand corner part is about the statistical results of different lithium concentration group (*** $P < 0.001$, or * $P < 0.05$). The abscissa was different lithium concentration; the ordinate was the relative extent of MLC_{20} phosphorylation. ($\bar{x}$±SD, n=8).

Fig.2 Effect of lithium on the amplitude of smooth muscle contraction in different incubation time

The top right-hand corner part is the diagram of scope and frequency curves of smooth muscle contraction. The arrow showed that the contraction scope and frequency were changed under the 20mM lithium concentration in different incubation time respectively. The excel picture of top left-hand corner part is about the statistical results of different incubation time group (*** $P < 0.001$, or * $P < 0.05$). The abscissa was different lithium concentration; the ordinate was the relative extent of MLC_{20} phosphorylation. ($\bar{x}$±SD, n=8).

Fig.3 Effect of lithium on MLC_{20} phosphorylation by MLCK in different lithium concentrations

The lane 0-4 in Fig.3 shows the negative control (blank control without incubation), positive control (without lithium) ,5, 10, 20mM lithium concentration group in the same incubation time respectively. i.e., the 0, 0, 5, 10, 20mM lithium were added into the lane 0, 1, 2, 3, 4 respectively for MLC_{20} phosphorylation. 4μM myosin (1.76mg·ml^{-1}) were used at 25℃ 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 in a Glycerol PAGE. Their mobilities were in the following order: myosin with unphosphorylated L_{20} (L_{20}) < mono-phosphorylated L_{20} (p-L_{20}) < myosin with di-phosphorylated 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 mono-phosphorylated 20 KDa myosin light chain; pp-MLC_{20} represents di-phosphorylated 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 * $P < 0.05$) ($\bar{x}$±SD, n=8).

Fig.4 Effect of lithium on MLC_{20} phosphorylation by MLCK in different incubation time

The lane 0-4 in Fig.3 shows the negative control (blank control without incubation), positive control (without lithium), 5, 10, 20min incubation time group in the 20mM lithium concentration respectively.

MLC_{20} phosphorylation is showed in Fig.3. Different incubation times i.e., lane 0' = 0 min, lane 1' = 20 min (without lithium), lane 2' = 5 min(with 20mM lithium), lane 3' = 10 min(with 20mM lithium), lane 4' = 20 min(with 20mM lithium) were chosen for MLC_{20} phosphorylation. 4μM myosin

($1.76 mg \cdot ml^{-1}$) were used at 25℃. MLC_{20} represents unphosphorylated 20 KDa myosin light chain; p-MLC_{20} represents mono-phosphorylated 20 KDa myosin light chain; pp-MLC_{20} represents di-phosphorylated 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. The t test value is described as: x±SD, n=8, *** = $P < 0.001$ and * = $P < 0.05$ compared to the corresponding controls.

Fig. 5 Effect of different concentration lithium on myosin Mg^{2+}-ATPase activities among MLC_{20} phosphorylation by MLCK in different incubation time course

The curve◆, ■, ▲and ● represents the Mg^{2+}-ATPase activities in groups with 20mM, 10mM, 5mM lithium and negative group respectively. 4μM myosin and 2μM MLCK were used in the assay in different incubation time, i.e., 5, 10, 20min. *t* test value is described as: (x±SD, n=8). &&&= P<0.001 compared with other groups with 5, 10, 20mM lithium; **= $P < 0.01$ compared. ***= $P<0.001$ compared with 5mM lithium; ##= $P < 0.01$ compared with 20mM lithium. ###= $P < 0.001$ compared with 20mM lithium.

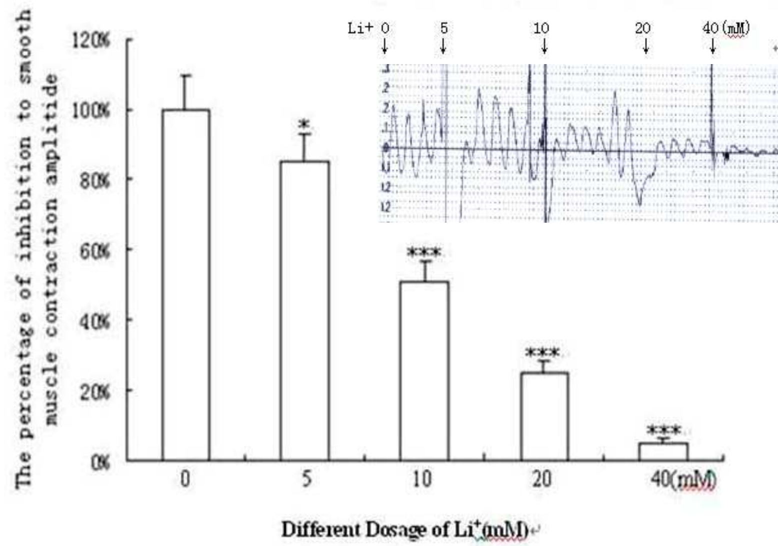

Fig. 1 Effect of lithium on the amplitude of smooth muscle myosin in different lithium concentration ($\bar{x}\pm$s, n=8).

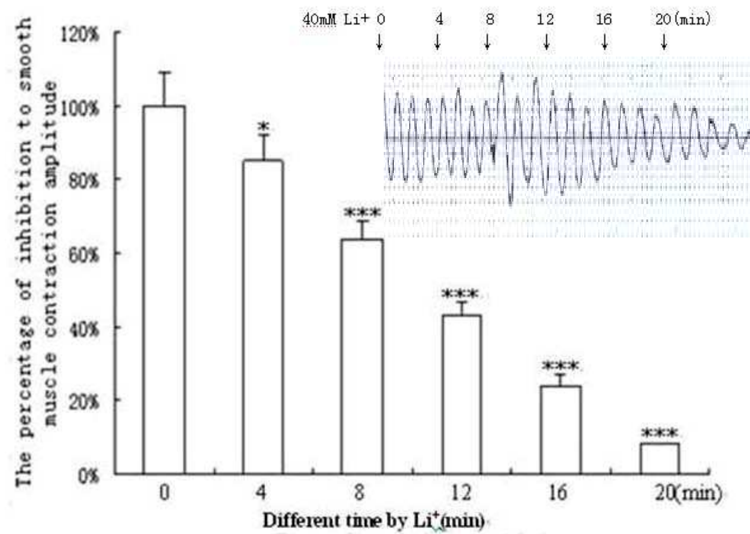

Fig.2 Effect of lithium on the amplitude of smooth muscle myosin in different incubation time($\bar{x}\pm$s, n=8).

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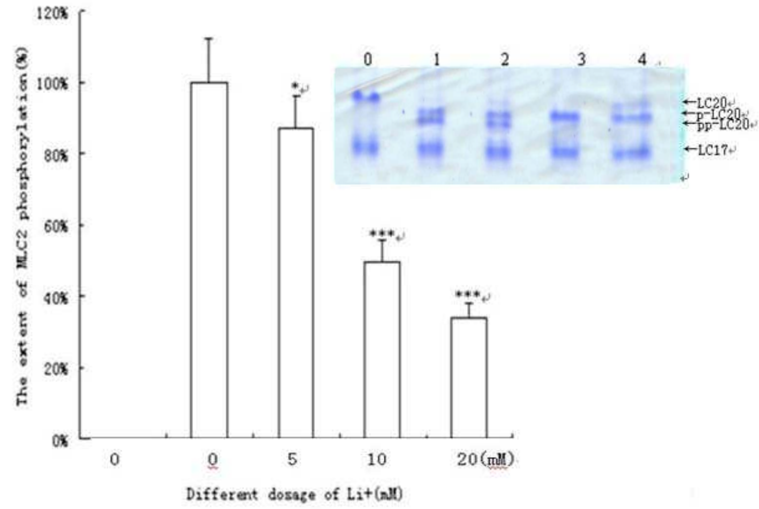

Fig.3 Effect of lithium on MLC_{20} phosphorylation by MLCK in different lithium concentrations ($\bar{x} \pm s$, n=8).

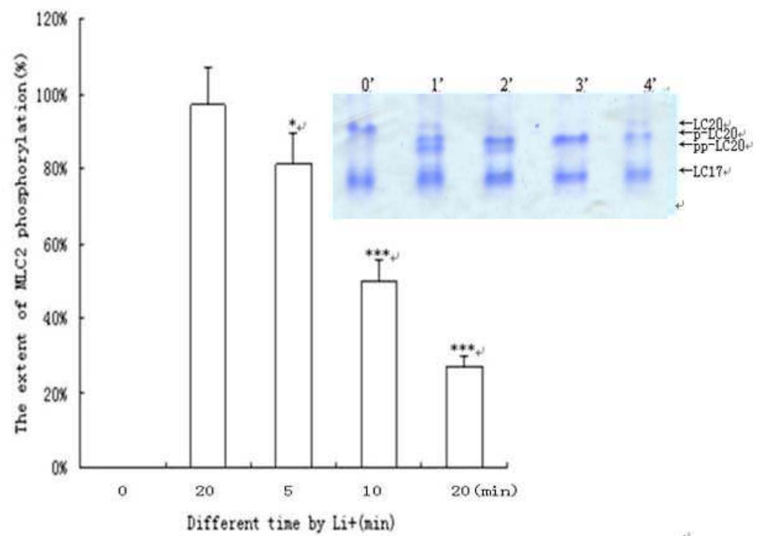

Fig.4 Effect of lithium on MLC_{20} phosphorylation by MLCK in different incubation time($\bar{x} \pm s$, n=8).

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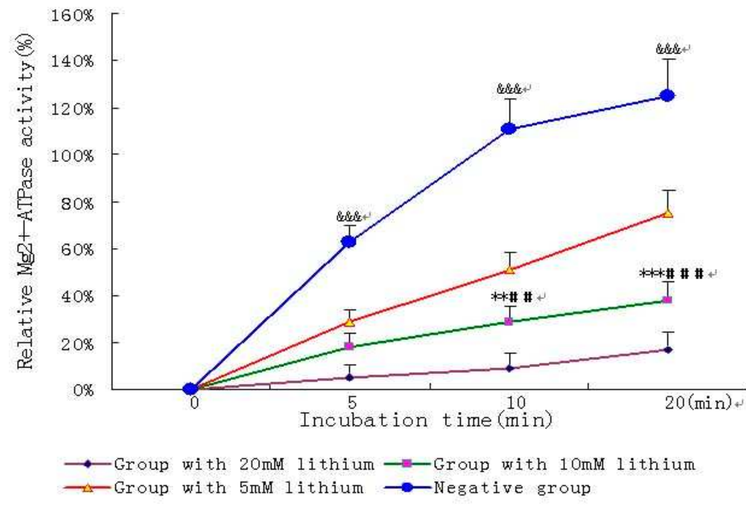

Fig.5 Effect of lithium on myosin Mg^{2+}-ATPase activities by 20mM, 10mM, 5mM lithium and negative group in different incubation time respectively ($\bar{x} \pm s$, n=8).

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