Published in final edited form as:

Int Urol Nephrol. 2016 August ; 48(8): 1237–1242. doi:10.1007/s11255-016-1303-2.

Carbachol-Induced Signaling through Thr696-Phosphorylation of Myosin Phosphatase Targeting Subunit 1 (MYPT1) in rat Bladder Smooth Muscle Cells

Benchun Liu^{#1}, Yung-Chin Lee^{#1,2}, Amjad Alwaal¹, Guifang Wang¹, Lia Banie¹, Ching-Shwun Lin¹, Guiting Lin¹, and Tom F. Lue^{*,1}

¹Knappe Molecular Urology Laboratory, Department of Urology, School of Medicine, University of California, San Francisco, CA, USA

²Department of Urology, Kaohsiung Medical University Hospital, Department of urology, Faculty of medicine, College of medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

These authors contributed equally to this work.

Abstract

Purpose—Lines of evidence suggest that Rho-associated protein kinase (ROCK) mediated myosin phosphatase targeting subunit 1 (MYPT1) phosphorylation play a central role in smooth muscle contraction. However, the physiological significance of MYPT1 phosphorylation at Thr696 catalyzed by ROCK in bladder smooth muscle remains controversial. We attempt to directly observe the quantitative protein expression of RhoA/ROCK and phosphorylation of MYPT1 at Thr696 after carbachol administration in rat bladder smooth muscle cells (RBMSCs).

Materials and Methods—Primary cultured smooth muscle cells were obtained from rat bladders. The effects of both concentration and time-course induced by the muscarinic agonist carbachol were investigated by assessing the expression of Rho A/ROCK and MYPT1 phosphorylation at Thr696 using Western blot.

Results—In the dose-course studies, carbachol showed significant increase of phosphorylation of MYPT1 at Thr696 (p-MYPT1) from concentrations of 15 μ M to 100 μ M based on Western blot results ($p < 0.05$, ANOVA test). In the time-course studies, treatment of cells with 15 μ M of carbachol significantly enhanced the expression of p-MYPT1 from 3 to 15 hr ($p < 0.05$, ANOVA test) and induced the expression of Rho A from 10 to 120 min ($p < 0.05$, ANOVA test).

Conclusions—Carbachol can induce the expression of ROCK pathway, leading to MYPT1 phosphorylation at Thr696 and thereby sustained RBSCs contraction.

*Correspondence to: Tom F. Lue, MD, Department of Urology, University of California, San Francisco, 400 Parnassus Ave., Ste A-610, San Francisco, CA 94143-0738, USA, Phone (O): 415-4761611, Fax: 415-476 8849, tlue@urology.ucsf.edu.

Conflict of interest: All the authors have declared no conflict of interest.

Ethical approval: This study was approved by the Institutional Animal Care and Use Committee of University of California San Francisco.

Keywords

bladder smooth muscle cell; carbachol; phosphorylation; rat; Rho-associated protein kinase (ROCK)

INTRODUCTION

The musculature of urinary bladder wall is primarily composed of smooth muscle cells. An initial development of force enables bladder to implement quick contractile responses, but they also may maintain force for an extended period of time to empty the bladder [1]. Adequate bladder contraction ensures complete urine emptying, while abnormal contractile performance of bladder smooth muscle can contribute to various diseases, such as urinary incontinence, overactive bladder or retention of urine [2,3]. Thus, in order to improve the clinical treatment of diseases related to voiding dysfunction, greater understanding of the signal transduction pathways involved in regulation of bladder smooth muscle contraction is essentially important.

Smooth muscle contraction is evoked by a network of signals involving a variety of membrane receptors and ion channels. Force production and maintenance in smooth muscle is primarily regulated by phosphorylation of the myosin light chain (MLC) [4]. It is well accepted that phosphorylation of the MLC catalyzed by the Ca^{2+} /calmodulin-dependent MLC kinase (MLCK) and dephosphorylation catalyzed by MLC phosphatase (MLCP) play a primary role in the regulation of smooth muscle contraction and relaxation [5,6]. MLCP is a heterotrimer that consists of three subunits: a 110-kDa regulatory myosin phosphatase-targeting subunit (MYPT1), a 38-kDa catalytic subunit of type 1 phosphatase (PP1c), and a 20-kDa subunit (M20) with unknown function [7]. Lines of evidence suggest that the regulatory MYPT1 subunit play the central role in the inhibition of cellular MLCP, which is essential for smooth muscle contraction [6,8]. Moreover, the inhibition of MLCP activity by Rho-associated protein kinase (ROCK) mediated MYPT1 phosphorylation is thought to act a key role in Ca^{2+} -sensitized contractions of different smooth muscles [9].

Upon muscarinic agonist stimulation, there are two major phosphorylation sites of MYPT1 which have been suggested to inhibit the activity of MLCP: Thr696 and Thr850 [10, 11]. Previous investigation indicated that carbachol stimulation could activate ROCK, which is thought to be the primary kinase to catalyze Thr696- and Thr850- phosphorylation of MYPT1. However, it is still controversial as to whether both Thr696- and Thr850-MYPT1 are endogenous targets of ROCK, depends on the smooth muscle tissue and the agonist used in the stimulation [12]. Moreover, there has been abundant evidence that phosphorylation of Thr850 catalyzed by ROCK occurs in a variety of smooth muscle tissues in response to diverse stimuli, the physiological importance of Thr696 phosphorylation by ROCK remains uncertain [13-15]. On the other hand, although an important role for modulation of MLC phosphorylation by MYPT1 is implicated by numerous biochemical studies in different smooth muscles, insights into the quantitative and integrative relationships among the signaling molecules acting, especially the prolonged effects of pharmacological agents, on bladder smooth muscle are still very limited.

To address these knowledge deficits, we carried out a study to investigate the effects of both concentration and time-course induced by the muscarinic agonist carbachol in rat bladder smooth muscle cells (RBSMCs). We directly observe the quantitative protein expression of ROCK and phosphorylation of MYPT1 at Thr696 by Western blot analysis.

MATERIALS AND METHODS

Cell culture and antibodies

This study was approved by the Institutional Animal Care and Use Committee of University of California San Francisco. Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). Fresh bladder tissues were obtained from female rats. RBSMCs were isolated as previously described [16]. Briefly, after washing extensively with phosphate buffered saline, bladder detrusor tissues were minced into pieces (1.5 to 2.0 mm³) in 10mm diameter culture dish, maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Inc., St. Louis, MO, USA) at 37 °C in a humidified incubator at an atmosphere of 5% CO₂. Primary cultures of RBSMCs were established in about two weeks. The cells were subcultured with the ethylenediaminetetraacetic acid (EDTA)–trypsin treatment when near confluence.

Carbamoylcholine chloride (carbachol, Sigma Aldrich Inc., St. Louis, MO, USA), a cholinomimetic drug that directly activates acetylcholine receptors, was dissolved in sterile water as stock solution and stored in 4°C. An appropriate volume of stock solution was diluted directly into the fresh culture medium and then added to each well or dish as a treatment.

For treatment, cells were plated at 1×10^5 cells per well in 6-well plates with 3 ml medium, or 10×10^5 cells per 100 mm diameter culture dish with 10 ml medium. After 24 hr incubation, the cells were treated with 10% FBS-DMEM with or without carbachol.

Mouse antibodies for MYPT1 were used as primary antibodies and RhoA-binding kinase was used for ROCK-II/ROK α . Rho proteins (1:500, BD Biosciences Pharmingen, Inc., San Diego, CA, USA), rabbit polyclonal antibody for anti-phospho-MYPT1 (Thr696) (1:1000, Upstate Inc., Lake Placid, NY, USA) and mouse monoclonal antibody for anti- β -actin (1:2000, Sigma Aldrich Inc., St. Louis, MO, USA) were commercially obtained. The secondary antibodies used were goat anti-rabbit Texas Red linked IgG (1:500, Vector Laboratories, Burlingame, CA, USA), in addition to peroxidase-conjugated secondary antibodies for goat anti-mouse, mouse anti-goat, and goat anti-rabbit IgG, which were obtained from the same company (1:500, Jackson immunoResearch laboratories, West Grove, PA, USA).

Protein isolation and Western blot analysis

The cellular protein samples from each time point were prepared by homogenization of cells in a lysis buffer containing 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium docetyl sulfate, aprotinin (10 µg/ml), leupeptin (10 µg/ml), and PBS. Cell lysates containing 20 µg of protein were electrophoresed in sodium docetyl sulfate polyacrylamide gel

electrophoresis and then transferred to a polyvinylidene fluoride membrane (Millipore Corp, Bedford, MA, USA). The membrane was stained with Ponceau S to verify the integrity of the transferred proteins and to monitor the unbiased transfer of all protein samples. Detection of target proteins on the membranes were performed with an electrochemiluminescence kit (Amersham Life Sciences Inc, Arlington Heights, IL, USA) with the use of primary antibodies for Rho A, ROCK, phospho-MYPT1 and β -actin. After the hybridization of secondary antibodies, the resulting images were analyzed with ChemiImager 4000 (Alpha Innotech Corporation, San Leandro, CA, USA) to determine the integrated density value of each protein band.

Statistical analysis

Data was shown as mean $\pm$ standard error of the mean (S.E.M.) of n experiments. Statistical analyses were carried out using one-way analysis of variance (ANOVA) for comparing multiple groups followed by Student Newman-Keuls test for two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Dose-course effects of carbachol on MYPT1 phosphorylation at Thr696

The phosphorylation of MYPT1 (p-MYPT1) at Thr696 was assessed by Western blot with different concentrations of carbachol for 2 hours. In the dose-course studies, the total protein level of MYPT1 remained constant after carbachol treatment. However, carbachol significantly enhanced the phosphorylation level of p-MYPT1 at Thr696 from 15 μ M to 100 μ M (15 μ M *vs.* control; 50 μ M *vs.* control; 100 μ M *vs.* control; all $p < 0.05$, ANOVA test). Expression of p-MYPT1 reached the peak level at 15 μ M (Fig. 1).

Time-course effects of carbachol on MYPT1 phosphorylation at Thr696

The expression of p-MYPT1 at Thr696 in RBSMCs was assessed by Western blot with carbachol treatment at 15 μ M from 1 to 15 hr. In the time-course studies, the total protein level of MYPT1 also remained constant. Carbachol significantly enhanced the phosphorylation level of p-MYPT1 from 3 to 15 hr based on Western blot results (3 hr *vs.* control; 6 hr *vs.* control; 15 hr *vs.* control; all $p < 0.05$, ANOVA test) and peaked at 6 hours time point post the treatment (Fig. 2).

Carbachol activated RhoA/ROCK pathway in RBSMCs

In the time-course studies of Rho A and ROCK expression with carbachol treatment at 15 μ M from 10 to 120 min. Carbachol significantly induced the expression of Rho A from 10 to 120 min (10 min *vs.* control; 30 min *vs.* control; 60 min *vs.* control; 120 min *vs.* control; all $p < 0.05$, ANOVA test) and the expression of Rho A reached the peak level at 10 min time point. Compared to controls, there were stepwise increases of ROCK I&II expressions from 10 to 60 min in the time-course studies, but the increases of expression did not reach the statistical significance ($P > 0.05$, ANOVA test) (Fig. 3)

DISCUSSION

The muscarinic agonist-stimulated smooth muscle contraction has two major regulatory mechanisms, including the activation of MLCK that phosphorylates MLC and the inhibition of MLCP activity that results in a net increase in MLC phosphorylation levels and therefore force. Historically, most studies have been aimed at the regulation of the MLCK as it was assumed that the MLCP was simply a constitutively active enzyme. However, it is now known that the MLCP is a regulated enzyme and plays an important role in the control of smooth muscle contraction [11]. Two signaling pathways are suggested to account for the muscarinic agonist-dependent inhibition of MLCP activity in smooth muscle contraction. The first is inhibition of the catalytic subunit PP1c by a small phosphorylatable inhibitory protein of 17 kDa (CPI-17) catalyzed by protein kinase C (PKC), while the second is ROCK-catalyzed phosphorylation of the regulatory subunit MYPT1 [9,17]. In this study, we investigated the effects of both concentration and time-course expression of phosphorylation pattern of MYPT1 as well as Rho A/ROCK induced by muscarinic agonist carbachol stimulation in primary RBSMCs. Our results demonstrated that carbachol can induce the activation of ROCK pathway, leading to MYPT1 phosphorylation at Thr696 and thereby sustained RBSMCs contraction.

In contrast to tonic vascular smooth muscle cells, phasic smooth cells such as urinary bladder has less CPI-17 protein [18]. Previous study reported that global deletion of MYPT1 causes embryonic lethality [19], suggesting a critical role of MYPT1-regulated MLCP during early developmental processes. As the main regulatory subunit of MLCP, MYPT1 appears to regulate MLCP activity biochemically through multiple mechanisms, such as serving as a binding subunit, phosphorylation at various sites affecting phosphatase activity and scaffolding with different proteins [6,20]. On the other hand, MYPT1 is a primary PP1c-binding protein in smooth muscle where it regulates the activity of MLCP holoenzyme by localizing the catalytic subunit to myosin filaments [21]. MYPT1 also can change the conformation of PP1c to increase its sensitivity for binding CPI-17 phosphorylated by PKC, thereby further inhibiting MLCP activity [22].

Although functional and biochemical studies suggest that ROCK mediated MYPT1 phosphorylation play a central role in smooth muscle contraction. The physiological significance of MYPT1 Thr696 phosphorylation catalyzed by ROCK as well as its functional roles are still controversial. It has been reported that muscarinic agonist can stimulate an increase in Thr696-MYPT1 phosphorylation in rat ileal smooth muscle tissues and cultured vascular aorta smooth muscle cells [23]. In terms of bladder smooth muscle, Mizuno et al. [24] reported that carbachol stimulation increased MYPT1 Thr696 phosphorylation in mice, being consistent with our current observations. More recently, a report from Khasnis et al [8] demonstrated that selective phosphorylation of MYPT1 at Thr696 with ROCK inhibited the MLCP activity 30%, whereas the Thr853 phosphorylation did not alter the phosphatase activity. Their results suggested that phosphorylation of Thr696 was more stable compared with that of Thr853, and it may facilitate Thr853 phosphorylation. However, investigations from Chen et al [25] indicated that MYPT1 T852 and CPI-17 are phosphorylated by muscarinic receptor stimulation, which could be reduced by ROCK and PKC inhibitors respectively, and that most MYPT1 Thr694 was

phosphorylated constitutively, similar to other reports for bladder smooth muscle [15,26]. So even in studies using the same smooth muscle preparation, the results can vary depending on the laboratory reporting the findings. Some of these controversial results may be due to differences of species, agonist employed and the techniques used to measure MYPT1 phosphorylation.

Previous reports have shown that MYPT1 Thr696 phosphorylation is not always increased by contractile agonists [27,28]. Basal Thr696 phosphorylation levels may be nearly maximal, and any further increase in Thr696 phosphorylation may require strong sustained stimulation. Compared to endogenous cholinergic neurotransmission, bath-applied Ach agonist may deliver a stronger signal that activates ROCK and has been reported to phosphorylate MYPT1 at Thr696 [29]. This finding was consistent with our current results that carbachol-stimulated can increase in Thr696 phosphorylation of MYPT1 by the ROCK pathway. However, it should be noted that several other kinases have been suggested to phosphorylate MYPT1 at Thr696. For example, p21-activated protein kinase and Raf-1 have been suggested to catalyze MYPT1 Thr696 phosphorylation and inhibit MLCP activity in vitro [30,31]. Integrin-linked kinase and ZIP-like kinase were also reported to be potential kinases that phosphorylate MLCP in vivo [32,33]. However, further investigations are required to clarify whether any of these kinases regulate the basal phosphorylation level of Thr696-MYPT1 in bladder smooth muscle.

In summary, our results have elucidated that the mechanisms involved in carbachol-induced bladder smooth muscle contraction is highly dependent on ROCK-catalyzed phosphorylation of MYPT1 at Thr696 in RBSMCs. These findings give significant insight into the importance of ROCK in regulation of bladder smooth muscle contraction induced by pharmacomechanical coupling mechanisms, and support the exploitation of ROCK agonists and inhibitors for the treatment of diseases related to voiding dysfunction. However, there are still some potential limitations needed to be addressed in this study. Firstly, although in vitro cell culture is a valuable technique for manipulating individual cells in a controlled experimental environment to study cell signaling related to various exogenous stimuli, smooth muscle cells grown in culture could have decrease expression of smooth muscle-specific proteins and the well-documented ability of smooth muscle cells could be modulated to noncontractile or migratory/secretory cells after a few subcultures. These changes that occur following culture of BSMCs may complicate the interpretation of our results obtained in vitro. Secondly, ROCK is the major kinase to be responsible for phosphorylation of the MYPT1 subunit of MLCP. However, PKC-catalyzed phosphorylation could potentially be involved through cross talk with ROCK. To further clarify these pathways underlying the activation of smooth muscle contraction, more studies involved both kinases, and their downstream effectors as well as inhibitors, may be required. Finally, given that our results are focused on bladder smooth muscle, additional investigations are needed to extend observations to other kinds of phasic smooth muscles in addition to tonic smooth muscles.

REFERENCES

1. Zderic SA, Chacko S. Alterations in the contractile phenotype of the bladder: lessons for understanding physiological and pathological remodelling of smooth muscle. *J Cell Mol Med.* 2012; 16:203–217. [PubMed: 21707917]
2. Hypolite JA, Lei Q, Chang S, et al. Spontaneous and evoked contractions are regulated by PKC-mediated signaling in detrusor smooth muscle: involvement of BK channels. *Am J Physiol Renal Physiol.* 2013; 304:451–462.
3. Stav K, Shilo Y, Zisman A, Lindner A, Leibovici D. Comparison of lower urinary tract symptoms between women with detrusor overactivity and impaired contractility, and detrusor overactivity and preserved contractility. *J Urol.* 2013; 189:2175–2178. [PubMed: 23220247]
4. He WQ, Qiao YN, Peng YJ, et al. Altered contractile phenotypes of intestinal smooth muscle in mice deficient in myosin phosphatase target subunit 1. *Gastroenterology.* 2013; 144:1456–1465. [PubMed: 23499953]
5. Kitazawa TG. protein-mediated Ca²⁺-sensitization of CPI-17 phosphorylation in arterial smooth muscle. *Biochem Biophys Res Commun.* 2010; 401:75–78. [PubMed: 20833141]
6. Grassie ME, Moffat LD, Walsh MP, MacDonald JA. The myosin phosphatase targeting protein (MYPT) family: A regulated mechanism for achieving substrate specificity of the catalytic subunit of protein phosphatase type 1 δ . *Arch Biochem Biophys.* 2011; 510:147–159. [PubMed: 21291858]
7. Matsumura F, Hartshorne DJ. Myosin phosphatase target subunit: many roles in cell function. *Biochem. Biophys. Res. Commun.* 2008; 369:149–156. [PubMed: 18155661]
8. Khasnis M, Nakatomi A, Gumpfer K, Eto M. Reconstituted Human Myosin Light Chain Phosphatase Reveals Distinct Roles of Two Inhibitory Phosphorylation Sites of the Regulatory Subunit. MYPT1. *Biochemistry.* 2014; 53:2710–2719.
9. Qiao YN, He WQ, Chen CP, et al. Myosin Phosphatase Target Subunit 1 (MYPT1) Regulates the Contraction and Relaxation of Vascular Smooth Muscle and Maintains Blood Pressure. *J Biol Chem.* 2014; 289:22512–22523. [PubMed: 24951589]
10. Abrams P, Andersson KE, Buccafusco JJ, et al. Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. *Br J Pharmacol.* 2006; 148:565–578. [PubMed: 16751797]
11. Wang T, Kendig DM, Smollock EM, Moreland RS. Carbachol-induced rabbit bladder smooth muscle contraction: roles of protein kinase C and Rho kinase. *Am J Physiol Renal Physiol.* 2009; 297:1534–1542.
12. Velasco G, Armstrong C, Morrice N, Frame S, Cohen P. Phosphorylation of the Regulatory Subunit of Smooth Muscle Protein Phosphatase 1 μ at Thr850 Induces Its Dissociation from Myosin. *FEBS Lett.* 2002; 527:101–104. [PubMed: 12220642]
13. Johnson RP, El-Yazbi AF, Takeya K, Walsh EJ, Walsh MP, Cole WC. Ca²⁺ sensitization via phosphorylation of myosin targeting subunit at threonine-855 by Rho kinase contributes to the arterial myogenic response. *J Physiol.* 2009; 587:2537–2553. [PubMed: 19359365]
14. Seok YM, Azam MA, Okamoto Y, et al. Enhanced Ca²⁺-dependent activation of phosphoinositide 3-kinase class II α isoform-Rho axis in blood vessels of spontaneously hypertensive rats. *Hypertension.* 2010; 56:934–941. [PubMed: 20921425]
15. Wang T, Kendig DM, Smollock EM, Moreland RS. Carbachol-induced rabbit bladder smooth muscle contraction: roles of protein kinase C and Rho kinase. *Am J Physiol.* 2010; 297:1534–1542.
16. Lin CS, Liu X, Chow S, Lue TF. Cyclic AMP and cyclic GMP activate protein kinase G in cavernosal smooth muscle cells: old age is a negative factor. *BJU Int.* 2002; 89:576–582. [PubMed: 11942968]
17. Ito M, Nakano T, Erdodi F, Hartshorne DJ. Myosin phosphatase: structure, regulation and function. *Mol Cell Biochem.* 2004; 259:197–209. [PubMed: 15124925]
18. Gao N, Huang J, He W, Zhu M, Kamm KE, Stull JT. Signaling through Myosin Light Chain Kinase in Smooth Muscles. *J Biol Chem.* 2013; 288:7596–7605. [PubMed: 23362260]
19. Okamoto R, Ito M, Suzuki N, et al. The targeted disruption of the MYPT1 gene results in embryonic lethality. *Transgenic Res.* 2005; 14:337–340. [PubMed: 16145842]

20. Matsumura F, Hartshorne DJ. Myosin phosphatase target subunit: many roles in cell function. *Biochem Biophys Res Commun.* 2008; 369:149–156. [PubMed: 18155661]
21. Shin HM, Je HD, Gallant C, et al. Differential association and localization of myosin phosphatase subunits during agonist-induced signal transduction in smooth muscle. *Circ. Res.* 2002; 90:546–553. [PubMed: 11909818]
22. Khromov A, Choudhury N, Stevenson AS, Somlyo AV, Eto M. Phosphorylation-dependent autoinhibition of myosin light chain phosphatase accounts for Ca²⁺ sensitization force of smooth muscle contraction. *J Biol Chem.* 2009; 284:21569–21579. [PubMed: 19531490]
23. Ohama T, Hori M, Sato K, Ozaki H, Karaki H. Chronic treatment with interleukin-1 β attenuates contractions by decreasing the activities of CPI-17 and MYPT-1 in intestinal smooth muscle. *J Biol Chem.* 2003; 278:48794–48804. [PubMed: 14512413]
24. Mizuno Y, Isotani E, Huang J, Ding H, Stull JT, Kamm KE. Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo. *Am J Physiol Cell Physiol.* 2008; 295:358–364.
25. Chen CP, Chen X, Qiao YN, et al. In vivo roles for myosin phosphatase targeting subunit-1 phosphorylation sites T694 and T852 in bladder smooth muscle contraction. *Physiol.* 2015; 593:681–700.
26. Wang T, Kendig DM, Chang S, Trappanese DM, Chacko S, Moreland RS. Bladder smooth muscle organ culture preparation maintains the contractile phenotype. *Am J Physiol Renal Physiol.* 2012; 303:1382–1397.
27. Borysova L, Shabir S, Walsh MP, Burdyga T. The importance of Rho-associated kinase-induced Ca²⁺ sensitization as a component of electromechanical and pharmacomechanical coupling in rat ureteric smooth muscle. *Cell Calcium.* 2011; 50:393–405. [PubMed: 21839512]
28. Mori D, Hori M, Murata T, et al. Synchronous phosphorylation of CPI-17 and MYPT1 is essential for inducing Ca²⁺ sensitization in intestinal smooth muscle. *Neurogastroenterol Motil.* 2011; 23:1111–1122. [PubMed: 22004286]
29. Bhetwal BP, Sanders KM, An C, Trappanese DM, Moreland RS, Perrino BA. Ca²⁺ sensitization pathways accessed by cholinergic neurotransmission in the murine gastric fundus. *J Physiol* 591. 2013; 12:2971–2986.
30. Takizawa N, Koga Y, Ikebe M. Phosphorylation of CPI17 and myosin binding subunit of type 1 protein phosphatase by p21-activated kinase. *Biochem Biophys Res Commun.* 2002; 297:773–778. [PubMed: 12359219]
31. Broustas CG, Grammatikakis N, Eto M, Dent P, Brautigan DL, Kasid U. Phosphorylation of the myosin-binding subunit of myosin phosphatase by Raf-1 and inhibition of phosphatase activity. *J Biol Chem.* 2002; 277:3053–3059. [PubMed: 11719507]
32. MacDonald JA, Borman MA, Murányi A, Somlyo AV, Hartshorne DJ, Haystead TA. Identification of the endogenous smooth muscle myosin phosphatase-associated kinase. *Proc Natl Acad Sci.* 2001; 98:2419–2424. [PubMed: 11226254]
33. Murányi A, MacDonald JA, Deng JT, et al. Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. *Biochem J.* 2002; 366:211–216. [PubMed: 12030846]

Figure 1.

Dose-course effects of carbachol on the phosphorylation of MYPT1 (p-MYPT1) at Thr696 in RBSMCs with concentrations of 0.1 μ M to 100 μ M for 2 hours. A) represents the blots and B) represents the quantification results. Carbachol significantly enhanced the phosphorylation level of p-MYPT1 at Thr696 from 15 μ M to 100 μ M based on Western blot analysis (15 μ M vs. control; 50 μ M vs. control; 100 μ M vs. control; all $p < 0.05$, ANOVA test). Expression of p-MYPT1 reached the peak level at 15 μ M.

Figure 2.

Time-course effects of carbachol on the phosphorylation of MYPT1 at Thr696 in RBSMCs at 15 μ M from 1 to 15 hr. A) represents the blots and B) represents the quantification results. Treatment of cells with carbachol at 15 μ M enhanced phosphorylation level of p-MYPT1 from 3 to 15 hr (3 hr vs. control; 6 hr vs. control; 15 hr vs. control; all $p < 0.05$, ANOVA test). Expression of p-MYPT1 reached the peak level at 6 hours.

Figure 3.

Time-course effects of carbachol on the expressions of Rho A and ROCK in RBSMCs at 15 μ M from 10 to 120 min. A) represents the blots and B) represents the quantification results. Compared to controls, carbachol significantly induced the expression of Rho A from 10 to 120 min (10 min vs. control; 30 min vs. control; 60 min vs. control; 120 min vs. control; all $p < 0.05$, ANOVA test) and the expression of Rho A reached the peak level at 10 min time point. There were stepwise increases of ROCK I&II expressions from 10 to 60 min in the time-course studies, but the increases of expression did not reach the statistical significance ($P > 0.05$, ANOVA test) (Fig. 3).