

Effects of amitriptyline, a tricyclic antidepressant, on smooth muscle

reactivity of isolated rat trachea

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A short title: Amitriptyline and airway smooth muscle relaxation

Key Words: Rats, Airway smooth muscle, Contractile response, Amitriptyline, Rho-kinase pathway, Phosphatidylinositol response

Abstract

Purpose. This study was designed to investigate the action of amitriptyline, a tricyclic antidepressant, on airway smooth muscle reactivity and its underlying mechanisms.

Methods. In isolated rat trachea, isometric force was recorded to examine the effects of amitriptyline on contractile response to acetylcholine (ACh), electrical field stimulation (EFS), calyculin A (myosin light chain phosphatase inhibitor) and sphingosylphosphorylcholine (Rho-kinase activator, SPC). In addition, inositol monophosphate (IP₁) accumulation was measured to examine its effects on inositol 1,4,5-trisphosphate (IP₃) production during stimulation with ACh.

Results. Amitriptyline inhibited contractile responses to ACh, EFS, calyculin A, and SPC with IC₅₀ values (mean ± SD) of 4.3 ± 1.3 μM, 3.2 ± 1.6 μM, 256.4 ± 106.4 μM, and 98.2 ± 21.8 μM, respectively. In addition, amitriptyline (10 μM) eliminated the ACh (10 μM)-induced IP₁ accumulation.

Conclusion. The results suggest that amitriptyline does not influence the tracheal smooth muscle reactivity at clinical concentrations ($< 1 \mu\text{M}$), but attenuates the reactivity at supraclinical concentrations ($\geq 1 \mu\text{M}$). The attenuated response to ACh by amitriptyline is presumably due, at least in part, to inhibition of the phosphatidylinositol (PI) metabolism. The ability of amitriptyline to inhibit the calyculin A-induced contraction suggests that amitriptyline also inhibits the Ca^{++} -calmodulin-myosin light chain pathway independently of inhibition of the PI metabolism. Finally, the difference in the IC_{50} values for SPC-induced contraction and calyculin A-induced contraction suggests that amitriptyline may also inhibit the Rho-kinase pathway.

Introduction

Amitriptyline, a tricyclic antidepressant, is commonly prescribed for patients with depression who undergo the electroconvulsive therapy (ECT) with general anesthesia. In addition, amitriptyline is now widely used for the treatment of chronic and neuropathic pain conditions [1, 2]. To date, amitriptyline has been reported to have multiple pharmacological actions such as inhibition of neural reuptake of noradrenaline, serotonin and adenosine, blockade of α -adrenergic, cholinergic, and N-methyl-D-aspartate receptors, modulation of opioid receptor-mediated function, or inhibition of ion channel activities [3, 4]. Thus, it would be important for anesthesiologists to understand its multiple pharmacological actions on organ functions, as well as its known interactions with various other drugs such as adrenergic agonists.

Previous studies [5, 6] have reported that amitriptyline significantly influences airway smooth muscle reactivity. In asthmatics, amitriptyline improved its clinical status, as well as reduced medication requirements [5,

6]. *In vivo* study of guinea pigs, amitriptyline counteracted the bronchoconstrictor actions of histamine, serotonin and acetylcholine (ACh) [5]. However, the precise mechanisms behind the inhibitory action of amitriptyline on airway smooth muscle have not yet been fully clarified.

The lung is innervated by both the sympathetic and parasympathetic nervous systems, which entail the activation of adrenergic and muscarinic receptors, respectively, located on both the airway smooth muscle and autonomic nerves [7]. Specifically, activation of the postjunctional M_3 muscarinic receptor leads to airway constriction, while activation of the prejunctional M_2 muscarinic receptor results in airway relaxation. In addition, activation of the prejunctional and/or postjunctional β_2 -adrenergic receptors would lead to airway relaxation. Thus, ACh (the parasympathetic neurotransmitter) and noradrenaline (the sympathetic neurotransmitter) both would play a crucial role in the control of airway smooth muscle tone or reactivity.

In the present study with isolated rat trachea, we first examined the effects

of amitriptyline on contractile response to both exogenously applied and endogenously released (in response to electrical field stimulation, EFS) ACh, and found that amitriptyline attenuates the contractile response to both exogenously applied and endogenously released ACh. Thus, we next attempted to clarify the mechanisms behind the amitriptyline-induced airway relaxation, utilizing various pharmacological tools including guanethidine (sympathetic suppressor), calyculin A (myosin light chain phosphatase inhibitor), and sphingosylphosphorylcholine (Rho-kinase activator, SPC). Specifically, guanethidine, calyculin A, and SPC were used to examine possible involvement of the noradrenaline-reuptake mechanism, Ca^{++} -calmodulin-myosin light chain kinase (MLCK) pathway, and Rho-kinase pathway, respectively, in the amitriptyline-induced airway relaxation. In addition, inositol monophosphate (IP_1) accumulation was measured to examine whether amitriptyline attenuates the contractile response to ACh by inhibiting the phosphatidylinositol (PI) metabolism.

Materials and Methods

The studies were conducted under guidelines approved by the Animal Care Committee of Nagasaki University School of Medicine. Fifty-two male Wistar rats (Charles River, Yokohama, Japan) weighing 250-350 g were used for the experiments. The rats were anesthetized with pentobarbital (50 mgkg^{-1} , i.p.), and their tracheas were rapidly isolated.

Isometric tension measurement

Each trachea was cut into 3-mm-wide ring segments with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). We used the distal two or three rings of the trachea. The tracheal ring was suspended between two stainless steel hooks and placed in a 5-mL water-jacketed organ chamber (UFER[®] UC-5, Kishimotoika, Kyoto, Japan) containing Krebs-Henseleit (K-H) solution (mM composition: NaCl 118, KCl 4.7, CaCl_2 1.3, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 25, glucose 11, $\text{Na}_2\text{-EDTA}$ 0.05). The solution was continuously aerated with O_2 95%/CO₂ 5% at 37°C.

Isometric tensions were measured using an isometric transducer (UFER[®]

UC-5, Kishimotoika, Kyoto, Japan) and changes in isometric force were recorded using a MacLab system (Milford, MA, USA). The resting tension was periodically adjusted to 1.0 g during the equilibration period. The rings were washed every 15 min and re-equilibrated to baseline tension for 60 min (Time 0).

Electrical Field Stimulation

Electrical stimuli were generated by SEN-7203 stimulator (Nihon Kohden, Tokyo) and applied between two platinum electrodes. Pulses of 2 ms and 50 V were delivered at frequency of 25 Hz for 10 sec, and 15 min recovery period was allowed between successive trains.

Measurement of IP₁ accumulation

The modified technique of Brown et al [8] was used. Inositol 1,4,5-trisphosphate (IP₃) is rapidly degraded into inositol monophosphate (IP₁) and subsequently recycled back to phosphatidylinositol via free inositol. Lithium inhibits the conversion of IP₁ to inositol, and in the presence of Li⁺ the accumulation rate of IP₁ reflects the extent of PI response.

We measured [^3H]IP₁ in the tracheal slices incubated with [^3H]myo-inositol (Amersham, Tokyo, Japan). Each trachea was longitudinally cut and chopped into 1-mm-wide slices with a McIlwain tissue chopper. Three pieces of the tracheal slice were placed in small flat-bottomed tubes and preincubated for 15 min in K-H solution containing 10 mM LiCl and continuously aerated with O₂ 95%/CO₂ 5%. An aliquot of 0.5 μCi [^3H]myo-inositol was then added to each tube (final concentration of 0.1 μM in a 300 μL incubation volume). The tubes were then flushed with O₂ 95%/CO₂ 5%, capped, set in a shaking bath at 37°C and incubated for 30 min (Time 0). Amitriptyline, 10 μM , or none was added at Time 0, and 15 min later, ACh, 10 μM , was added. After 60 min, the reaction was stopped with 940 μL of chloroform: methanol (1: 2 v/v). Chloroform and water were then added (300 μL each) and the phases were separated by centrifugation at 90 g for 5 min. The [^3H]IP₁ was separated from [^3H]myo-inositol in the 750 μL water phase by column chromatography using Dowex AG 1-X8 resin (Bio Rad, Richmond, CA, USA) in its formate form. [^3H]IP₁ formed in the

tracheal slices was counted using a liquid scintillation counter.

Experimental protocols

In the first series of experiments, we examined the effects of amitriptyline on contractile response to exogenously applied ACh.

Specifically, amitriptyline (1 μ M - 1 mM) was cumulatively applied to the rings precontracted with ACh (10 μ M).

In the second series of experiments, we attempted to examine the effects of amitriptyline on contractile response to endogenously released ACh, utilizing contractile response to EFS. In preliminary experiments, both tetrodotoxin (TTX, a Na channel blocker [9] and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, a M_3 -cholinergic receptor antagonist [10] inhibited the EFS response with IC_{50} values of 20 ± 8 nM and 3.0 ± 0.8 nM, respectively, eliminated it at 100 nM and 30 nM, respectively (Figure 1). However, prazosin (0.1 - 10 μ M, α_1 -adrenergic blocker [11] and capsaicin (0.1 - 10 μ M, a neuronal desensitizer depleting calcitonin gene related peptide [CGRP] from the

nerve terminals [12])) did not influence the EFS response ($P > 0.05$, $n = 6$, not shown). These results suggest that the EFS response was mediated exclusively by ACh released from the nerve terminals, but not by α_1 -adrenergic receptors or CGRF. Also in this series of experiments, amitriptyline (1 - 100 μM) was cumulatively applied to the rings precontracted with EFS.

In the above experiments, amitriptyline significantly inhibited contractile response to ACh or EFS. Thus, in the following experiments, we attempted to clarify the mechanisms underlying the amitriptyline-induced tracheal relaxation, utilizing various pharmacological tools. Specifically, in the third series of experiments, in order to investigate a possibility that amitriptyline causes tracheal relaxation by inhibiting the reuptake of noradrenalin into the sympathetic nerves, we examined the effects of amitriptyline (1 - 100 μM) on contractile response to EFS after treatment with guanethidine (10 μM), which depletes noradrenaline from the sympathetic nerves [13].

In the fourth series of experiments, in order to investigate a possibility that

amitriptyline causes tracheal relaxation by inhibiting the Ca^{++} -calmodulin-MLCK pathway, we examined the effects of amitriptyline on contractile response to calyculin A (a MLC phosphatase inhibitor [14]). Specifically, amitriptyline (10 μM - 1 mM) was applied to the rings for 15 min before stimulation with calyculin A (1 μM), because the contractile response to calyculin-A ran down significantly with time in the preliminary study.

In the fifth series of experiments, in order to investigate a possibility that amitriptyline causes tracheal relaxation by inhibiting the Rho-kinase pathway, we examined the effects of amitriptyline on contractile response to SPC (a Rho-kinase activator [15]). Specifically, amitriptyline (10 μM – 1 mM) was cumulatively applied to the rings precontracted with SPC (10 μM).

In the final series of experiments, utilizing the Brown's [8] method detailed above, we examined the effects of amitriptyline on ACh-induced IP_1 accumulation. Amitriptyline (10 μM) or none was applied to the slices before stimulation with ACh (10 μM).

Data analyses

Data were expressed as mean \pm SD. Concentration-effect curves were fitted by nonlinear regression (GraphPad Prism; GraphPad, San Diego, CA, USA). The results for guanethidine data were analyzed by two-way ANOVA. Since the interaction was statistically significant, data between control and guanethidine group were analyzed by one-way ANOVA. The other results were also analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. A value of $P < 0.05$ was considered statistically significant.

Results

Amitriptyline inhibited ($P < 0.05$) the contractile response to ACh (10 μM) in a concentration-dependent manner with an IC_{50} value of 4.3 ± 1.3 μM ($n = 6$) (Figure 2B). In addition, amitriptyline inhibited ($P < 0.05$) the contractile response to EFS in a concentration-dependent manner with an IC_{50} value of 3.2 ± 1.6 μM ($n = 9$) (Figure 3). No significant difference was observed in the IC_{50} value for inhibition of ACh response and that for inhibition of EFS response.

Treatment with guanethidine (10 μM) caused ($P < 0.05$) a modest leftward shift of the concentration-response curve for the inhibitory action of amitriptyline on contractile response to EFS (Figure 3). The IC_{50} value for the amitriptyline-induced inhibition of EFS response (1.3 ± 0.4 μM , $n = 9$) in the guanethidine-treated rings was lower ($P < 0.05$) than in the control rings (3.2 ± 1.6 μM , $n = 9$).

Amitriptyline inhibited ($P < 0.05$) the contractile response to calyculin A (1 μM) in a concentration-dependent manner with an IC_{50} value of $256.4 \pm$

106.4 μM ($n = 7-11$) (Figure 4), and almost eliminated it at 300 μM .

Amitriptyline inhibited ($P < 0.05$) the contractile response to SPC (10 μM) in a concentration-dependent manner with an IC_{50} value of 98.2 ± 21.8 μM ($n = 8$) (Figure 5B), and almost eliminated it at 300 μM .

The basal IP_1 accumulation, in which no drugs was administered, was 494 ± 91 DPM. ACh (10 μM) increased the IP_1 accumulation to 914 ± 56 DPM, and amitriptyline inhibited ($P < 0.05$) the ACh-induced IP_1 accumulation to 421 ± 51 DPM (Figure 6).

Discussion

The present results indicate that amitriptyline attenuates contractile response to both exogenously applied and endogenously released ACh, the parasympathetic neurotransmitter that plays a central role in the regulation of airway smooth muscle tone *in vivo*. However, its effective concentrations were significantly higher than clinically relevant concentrations ($< 1 \mu\text{M}$). Thus, amitriptyline would not influence the contractile response to ACh through the peripheral (i.e., direct and peripheral nerve) action on airway smooth muscle in the normal clinical setting.

James et al. [16] observed that effects of tricyclic antidepressants on gastric smooth muscle contractility in guinea pig, and found that amitriptyline reduced contractions induced by ACh. Huang & Lau [17] reported that in the rat isolated trachea, amitriptyline produced a shift to the right of ACh concentration-contraction curve without affecting the maximum contraction. The IC_{50} value was $10.3 \mu\text{M}$ for inhibition of the submaximal contractile response to $1 \mu\text{M}$ ACh. This IC_{50} value is nearly

consistent with the value of our present study.

The most important mechanism for smooth muscle contraction is activation of the PI cascade as a result of interaction between a contractile molecule and a specific receptor coupled to phospholipase C (PLC). Specifically, the majority of contractile agonists acts on G-protein-coupled receptors, and thereby activate PLC to hydrolyze the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into IP₃ and diacylglycerol (Figure 7). IP₃ stimulates the sarcoplasmic reticulum to release Ca⁺⁺ that in turn activates the Ca⁺⁺-calmodulin-MLCK pathway and initiates contraction (Figure 7), while diacylglycerol activates protein kinase C, possibly leading to an increase in the myofilament Ca⁺⁺ sensitivity. Following the initial increase in [Ca⁺⁺]_c caused by the IP₃-induced Ca⁺⁺ release, a tonic increase in [Ca⁺⁺]_c, which is largely dependent on extracellular Ca⁺⁺, is normally observed in contractile response to receptor agonists. In previous studies with isolated human mesenteric arteries [18], human vas deference [19], rabbit aorta [20], or guinea-pig ileum [21, 22],

amitriptyline inhibited contractile response to various receptor agonists such as, noradrenalin [18-20], histamine [21], or serotonin [22]. These results suggest that amitriptyline may inhibit activation of the PI cascade and thereby attenuate smooth muscle contractile activity. Indeed, in this study, amitriptyline inhibited the ACh-induced IP_1 accumulation, providing the first direct evidence for the amitriptyline-induced inhibition of the ACh-induced IP_3 production (i.e., ACh-activation of the PI cascade). Amitriptyline presumably inhibits the activation of G protein-coupled PLC in the tracheal smooth muscle membrane.

It is generally believed that in humans, stimulation of β_2 -adrenergic receptors leads to airway relaxation through direct action on airway smooth muscle and/or presynaptic inhibition of the vagus nerve [23-25]. Since amitriptyline inhibits the reuptake of noradrenaline into nerve terminals in the brains [3], amitriptyline may also inhibit the reuptake of noradrenaline into nerve terminals in airway smooth muscle and thereby cause airway relaxation. However, treatment with guanethidine, which depletes

noradrenaline storage vesicles in the nerve terminal [26], did not inhibit but enhanced the amitriptyline-induced airway relaxation. Thus, inhibition of the reuptake of noradrenaline into nerve terminals would not be involved in the amitriptyline-induced airway relaxation. The precise mechanisms behind the enhancement are currently unknown, and would require further research.

When agonists stimulate receptors on airway smooth muscle cell membranes, Rho, a small G-protein, activates Rho-kinase, which in turn inactivates myosin phosphatase (Figure 7). Inactivation of myosin phosphatase increases myosin light chain phosphorylation, resulting in an increased contraction. The airway smooth muscle contraction occurs through activation of the receptors coupled with small G-proteins in canine, rabbit, and human airway smooth muscles *in vitro*, and involves Rho-kinase pathway [27-30]. It has been suggested that SPC causes vasoconstrictions by activating the Rho-kinase pathway in cerebral, coronary and pulmonary arteries [15, 31, 32]. In this study, amitriptyline inhibited the SPC-induced contraction, suggesting a possibility that amitriptyline inhibits the

Rho-kinase. However, amitriptyline also inhibited the calyculin A-induced contraction—i.e., contraction caused by inhibition of MLC phosphatase. Thus, the ability of amitriptyline to inhibit the SPC-induced contraction could be simply explained by its ability to inhibit the Ca^{++} -calmodulin-MLCK pathway. Nevertheless, we speculate that amitriptyline may inhibit the Rho-kinase, because the IC_{50} value for inhibition of the SPC-induced contraction ($98\ \mu\text{M}$) is significantly lower than that for inhibition of the calyculin A-induced contraction ($256\ \mu\text{M}$). Further research—including direct measurement of the Rho-kinase activity—is required to clarify this issue.

In conclusion, amitriptyline attenuates the reactivity at supraclinical concentrations ($\geq 1\ \mu\text{M}$). The attenuated response to ACh by amitriptyline is presumably due, at least in part, to inhibition of the PI metabolism. The ability of amitriptyline to inhibit the calyculin A-induced contraction suggests that amitriptyline also inhibits the Ca^{++} -calmodulin-MLCK pathway independently of inhibition of the PI metabolism. Finally, the

difference in the IC_{50} values for SPC-induced contraction and calyculin A-induced contraction suggests that amitriptyline may also inhibit the Rho-kinase pathway.

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References

1. Tremont-Lukats IW, Challapalli V, McNicol ED, Lau J, Carr DB.
Systemic administration of local anesthetics to relieve neuropathic pain:
a systematic review and meta-analysis. *Anesth Analg.*
2005;101:1738-49.
2. Haderer A, Gerner P, Kao G, Srinivasa V, Wang GK. Cutaneous
analgesia after transdermal application of amitriptyline versus lidocaine
in rats. *Anesth Analg.* 2003;96:1707-10.
3. Sawynok J, Esser MJ, Reid AR. Antidepressants as analgesics: an
overview of central and peripheral mechanisms of action. *J Psychiatry
Neurosci.* 2001;26:21-9.
4. Sawynok J, Reid AR, Liu XJ, Parkinson FE. Amitriptyline enhances
extracellular tissue levels of adenosine in the rat hindpaw and inhibits
adenosine uptake. *Eur J Pharmacol.* 2005;518:116-22.
5. Meares RA, Mills JE, Horvath TB, Atkinson JM, Pun LQ, Rand MJ.
Amitriptyline and asthma. *Med J Aust.* 1971; 2:25-8.

6. Wilson RC. Antiasthmatic effect of amitriptyline. *Can Med Assoc J.* 1974;111:212.
7. Proskocil BJ, Fryer AD. Beta2-agonist and anticholinergic drugs in the treatment of lung disease. *Proc Am Thorac Soc.* 2005;2:305-10.
8. Brown E, Kendoll DA, Nahorski SR. Inositolphospholipid hydrolysis in rat cerebral cortical slices: I. Receptor characterization. *J Neurochem.* 1984; 42:1379-87.
9. Toda N, Hatano Y. Contractile responses of canine tracheal muscle during exposure to fentanyl and morphine. *Anesthesiology.* 1980;53:93-100.
10. Itabashi S, Aikawa T, Sekizawa K, Ohru T, Sasaki H, Takishima T. Pre- and postjunctional muscarinic receptor subtypes in dog airways. *Eur J Pharmacol.* 1991;204:235-41.
11. Yu M, Wang Z, Robinson NE. Prejunctional α_2 -adrenoceptors inhibit acetylcholine release from cholinergic nerves in equine airways. *Am J Physiol.* 1993;265:L565-70.

12. Szarek JL, Spurlock B. Antagonism of cholinergic nerve-mediated contractions by the sensory nerve inhibitory system in rat bronchi. *J Appl Physiol.* 1996;81:260-5.
13. Lindén A, Ullman A, Skoogh BE, Löfdahl CG. The non-adrenergic, non-cholinergic response counteracts changes in guinea-pig airway tone with and without sympathetic activation. *Br J Pharmacol.* 1992;106:616-22.
14. Burdyga T, Mitchell RW, Ragozzino J, Ford LE. Force and myosin light chain phosphorylation in dog airway smooth muscle activated in different ways. *Respir Physiol Neurobiol.* 2003;137:141-9.
15. Nakao F, Kobayashi S, Mogami K, Mizukami Y, Shirao S, Miwa S, Todoroki-Ikeda N, Ito M, Matsuzaki M. Involvement of Src family protein tyrosine kinases in Ca^{2+} sensitization of coronary artery contraction mediated by a sphingosylphosphorylcholine-Rho-kinase pathway. *Circ Res.* 2002;91:953-60.
16. James AN, Ryan JP, Parkman HP. Effects of clonidine and tricyclic

- antidepressants on gastric smooth muscle contractility.
Neurogastroenterol Motil. 2004;16:143-53.
17. Huang Y, Lau CW. Inhibitory effect of amitriptyline on contraction of the rat isolated trachea. Pharmacology. 1997;54:312-8.
18. Vila JM, Medina P, Segarra G, Lluch P, Pallardo F, Flor B, Lluch S. Relaxant effects of antidepressants on human isolated mesenteric arteries. Br J Clin Pharmacol. 1999; 48:223-9.
19. Medina P, Segarra G, Ballester R, Chuan P, Domenech C, Vila JM, Lluch S. Effects of antidepressants in adrenergic neurotransmission of human vas deferens. Urology. 2000; 55:592-7.
20. Auguet M, Clostre F, DeFeudis FV. Effects of antidepressants on receptor-activated and Ca^{2+} -activated contractions of rabbit isolated aorta. Gen Pharmacol. 1986; 17:607-10.
21. Kachur JF, Allbee WE, Gagarella TS. Antihistaminic and antimuscarinic effects of amitriptyline on guinea pig ileal electrolyte

- transport and muscle contractility in vitro. *J Pharmacol Exp Ther.* 1988; 245:455-9.
22. Lucchelli A, Santagostino-Barbone MG, D'Agostino G, Masoero E, Tonini M. The interaction of antidepressant drugs with enteric 5-HT₇ receptors. *Naunyn Schmiedebergs Arch Pharmacol.* 2000; 362:284-9.
23. Thirstrup S. Control of airway smooth muscle tone: II-pharmacology of relaxation. *Respir Med.* 2000;94:519-28.
24. Thirstrup S. Control of airway smooth muscle tone. I-electrophysiology and contractile mediators. *Respir Med.* 2000;94:328-36.
25. Proskocil BJ, Fryer AD. Beta2-agonist and anticholinergic drugs in the treatment of lung disease. *Proc Am Thorac Soc.* 2005;2:305-10.
26. Wood M, Wood AJJ. *Drugs and anesthesia: pharmacology for anesthesiologists*, 2nd ed. Baltimore: Williams & Wilkins Inc.; 1990. p.443-4.
27. Iizuka K, Dobashi K, Yoshii A, Horie T, Suzuki H, Nakazawa T, Mori M. Receptor-dependent G protein-mediated Ca²⁺ sensitization in canine

- airway smooth muscle. *Cell Calcium*. 1997; 22:21-30.
28. Iizuka K, Yoshii A, Samizo K, Tsukagoshi H, Ishizuka T, Dobashi K, Nakazawa T, Mori M. A major role for the rho-associated coiled coil forming protein kinase in G-protein-mediated Ca^{2+} sensitization through inhibition of myosin phosphatase in rabbit trachea. *Br J Pharmacol*. 1999; 128:925-33.
29. Yoshii A, Iizuka K, Dobashi K, Horie T, Harada T, Nakazawa T, Mori M. Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. *Am J Respir Cell Mol Biol*. 1999; 20:1190-200.
30. Iizuka K, Shimizu Y, Tsukagoshi H, Yoshii A, Harada T, Dobashi K, Murozono T, Nakazawa T, Mori M. Evaluation of Y-27632, a rho-kinase inhibitor, as a bronchodilator in guinea pigs. *Eur J Pharmacol*. 2000; 406:273-9.
31. Thomas GD, Snetkov VA, Patel R, Leach RM, Aaronson PI, Ward JP. Sphingosylphosphorylcholine-induced vasoconstriction of pulmonary

artery: activation of non-store-operated Ca^{2+} entry. *Cardiovasc Res.*

2005; 68:56-64.

32. Shirao S, Kashiwagi S, Sato M, Miwa S, Nakao F, Kurokawa T, Todoroki-Ikeda N, Mogami K, Mizukami Y, Kuriyama S, Haze K, Suzuki M, Kobayashi S. Sphingosylphosphorylcholine is a novel messenger for Rho-kinase-mediated Ca^{2+} sensitization in the bovine cerebral artery: unimportant role for protein kinase C. *Circ Res.* 2002; 91:112-9.

Legends

Fig. 1.

The effects of tetrodotoxin (TTX) and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) on electrical field stimulation (EFS)-induced contraction of rat tracheal rings (mean \pm SD). Twelve tracheal rings (n = 12) from 6 rats (N = 6) were used in the experiments. ** $P < 0.01$; *** $P < 0.001$ vs drugs 0. EFS: 25 Hz.

Fig. 2A.

A recording of the effects of amitriptyline on acetylcholine (ACh)-induced contraction of the rat tracheal ring. ACh, 10 μ M was added, and 10 min later, tension was measured by stepwise cumulative additions of amitriptyline. ACh-induced contractions were observed to continue over 90 min in the preliminary study.

Fig. 2B.

The effects of amitriptyline on ACh-induced contraction of rat tracheal rings (mean \pm SD; n = 6, N = 3). ** $P < 0.01$; *** $P < 0.001$ vs amitriptyline

0. ACh: 10 μ M acetylcholine. Concentrations of amitriptyline to exert 50% inhibition (IC_{50}) of ACh-induced contractions were $4.3 \pm 1.3 \mu$ M.

Fig. 3.

The effects of amitriptyline on electrical field stimulation (EFS)-induced contraction of rat tracheal rings in the absence or presence of guanethidine (mean \pm SD; n = 9, N = 10). * $P < 0.05$, *** $P < 0.001$ vs Control. EFS: 25 Hz, Guanethidine: 10 μ M. Concentrations of amitriptyline to exert 50% inhibition (IC_{50}) of EFS-induced contractions were 3.2 ± 1.6 and $1.3 \pm 0.4 \mu$ M in the absence and presence of guanethidine respectively.

Fig. 4.

The effects of amitriptyline on calyculin A (myosin phosphatase inhibitor)-induced contraction of rat tracheal rings (mean \pm SD; n = 7-11, N = 15). Amitriptyline was added prior to calyculin A, because contraction induced by calyculin A, reached maximum within 30 min, followed by spontaneous decrease to the baseline in the preliminary study. *** $P < 0.001$ vs amitriptyline 0. Calyculin A: 1 μ M. Concentration of

amitriptyline to exert IC_{50} of calyculin A-induced contractions was $256.4 \pm 106.4 \mu\text{M}$.

Fig. 5A.

A recording of the effects of amitriptyline on SPC-induced contraction of the rat tracheal ring. SPC, $10 \mu\text{M}$ was added, and 30 min later, tension was measured by stepwise cumulative additions of amitriptyline. SPC-induced contractions were observed to continue over 90 min in the preliminary study.

Fig. 5B.

The effects of amitriptyline on SPC-induced contraction of rat tracheal rings (mean \pm SD; $n = 8$; $N = 6$). *** $P < 0.001$ vs amitriptyline 0. SPC: $10 \mu\text{M}$ sphingosylphosphorylcholine. Concentrations of amitriptyline to exert 50% inhibition (IC_{50}) of SPC-induced contractions were $98.2 \pm 21.8 \mu\text{M}$.

Fig. 6.

The effects of amitriptyline on IP_1 accumulation in rat tracheal slices (mean \pm SD; $n = 6-7$, $N = 8$). IP_1 : inositol monophosphate. DPM:

disintegration per minute. Amitriptyline (Ami): 10 μ M, Acetylcholine (ACh): 10 μ M. *** $P < 0.001$ vs basal. # $P < 0.001$ vs ACh.

Fig. 7.

A flow diagram of the phosphatidylinositol (PI) response and Rho-kinase pathway. G: G-protein, PI: phosphatidylinositol, PIP: phosphatidylinositol 4-phosphate, PIP₂: phosphatidylinositol 4,5-bisphosphate, IP₃: inositol 1,4,5 trisphosphate, IP₂: inositol bisphosphate, IP₁: inositol monophosphate, MLC: myosin light chain, P-MLC: phosphorylated myosin light chain.

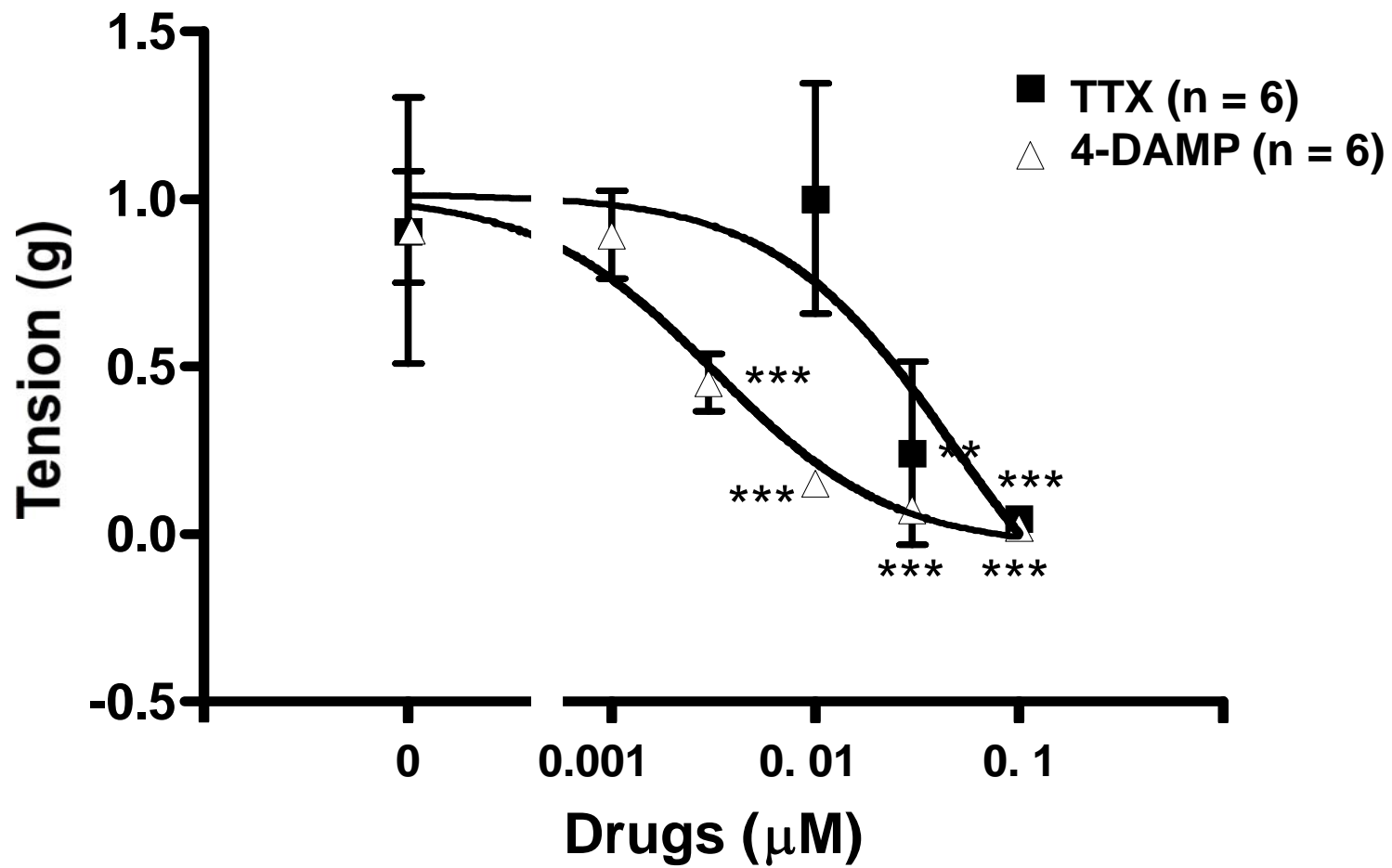


Figure 1

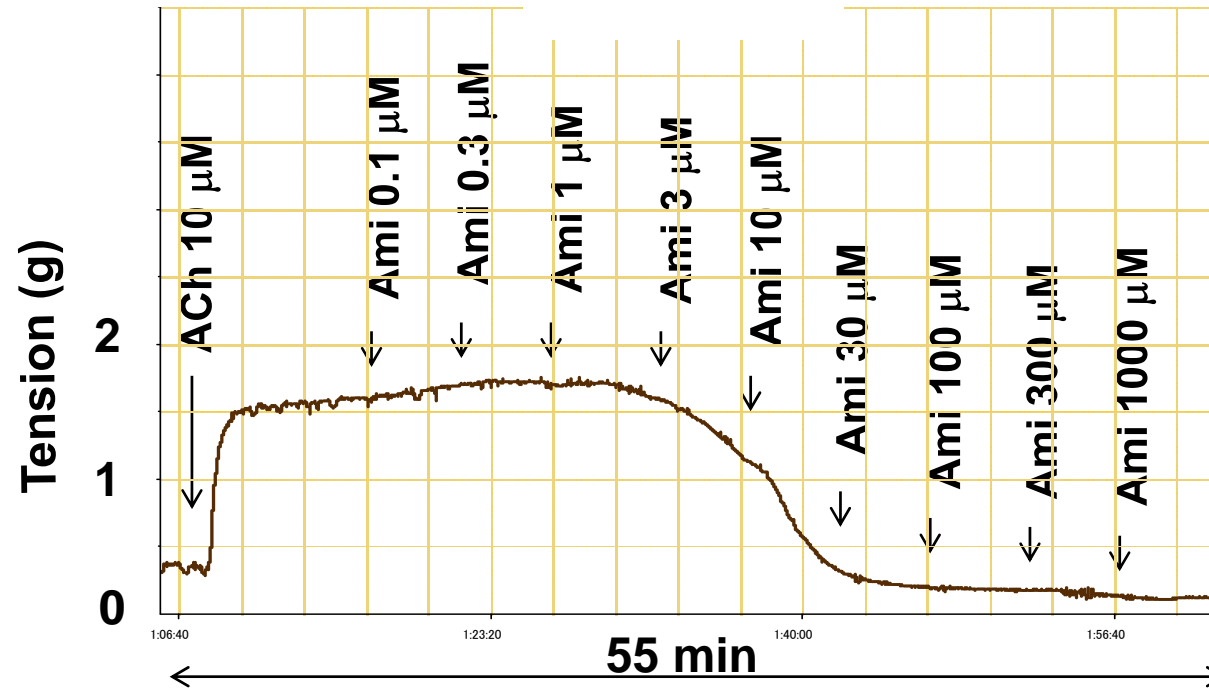


Figure 2A

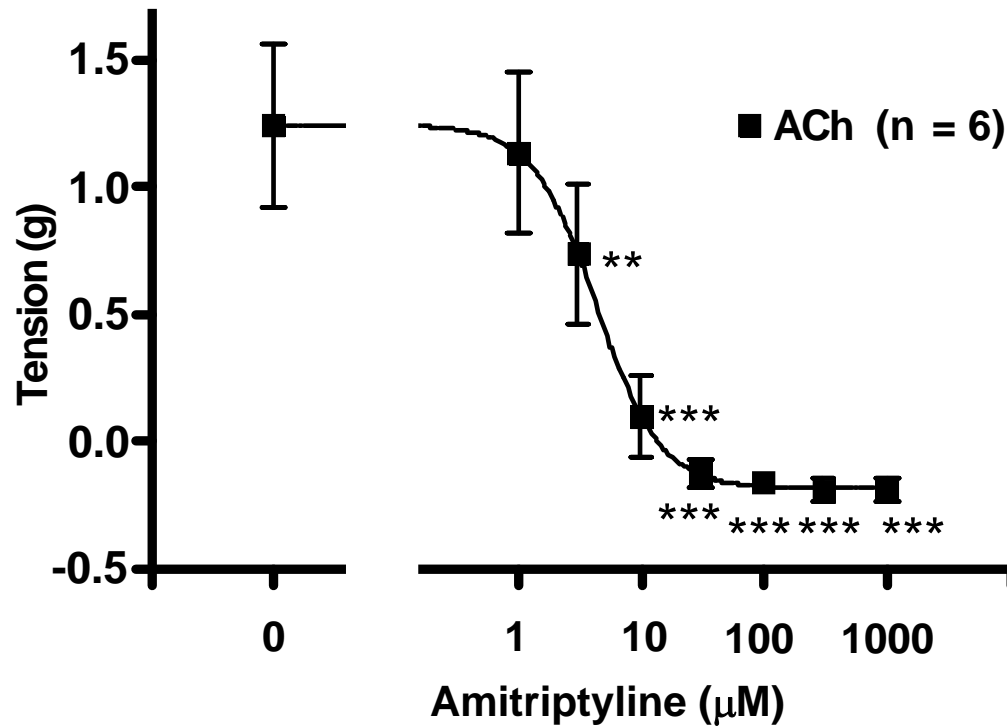


Figure 2B

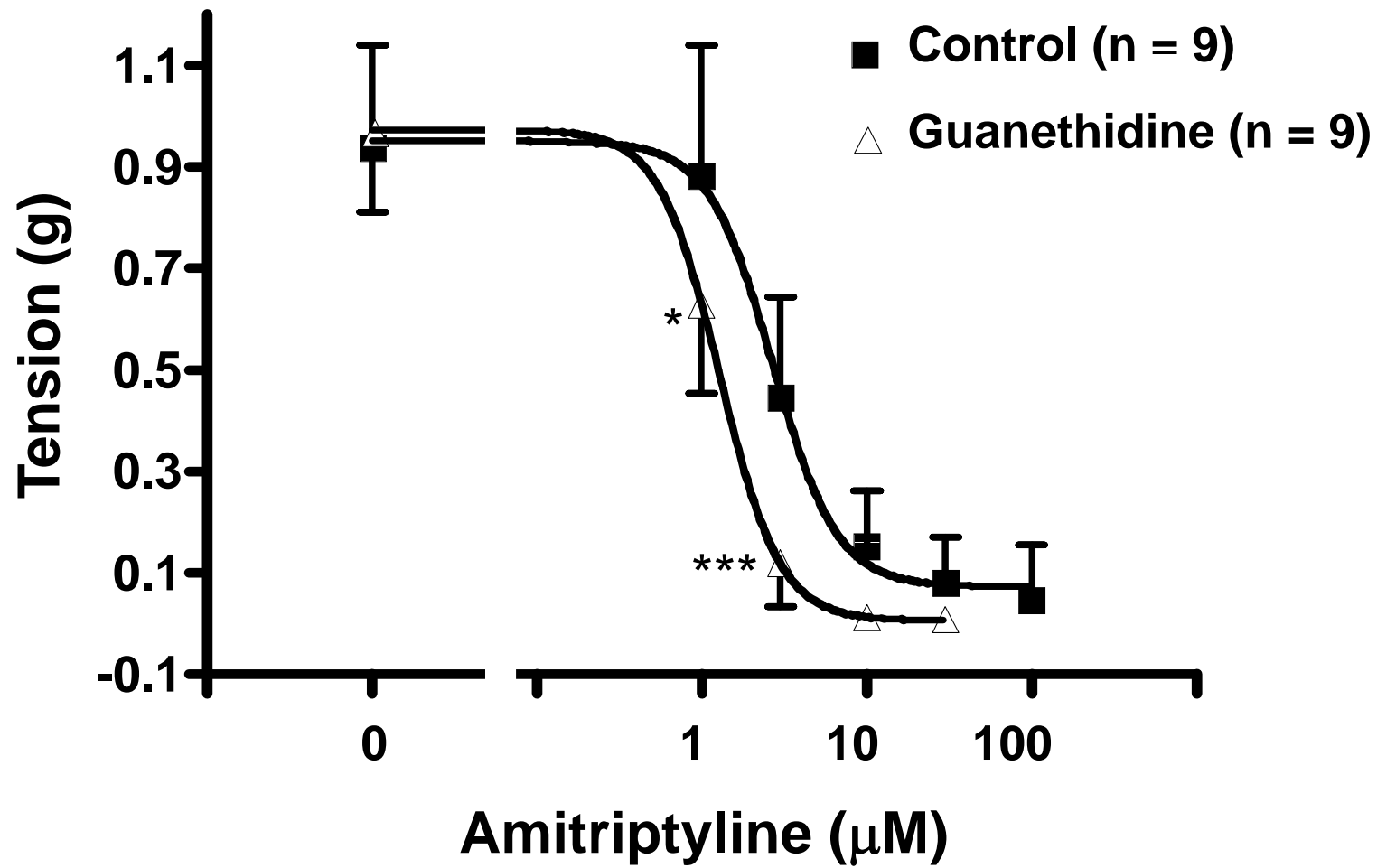


Figure 3

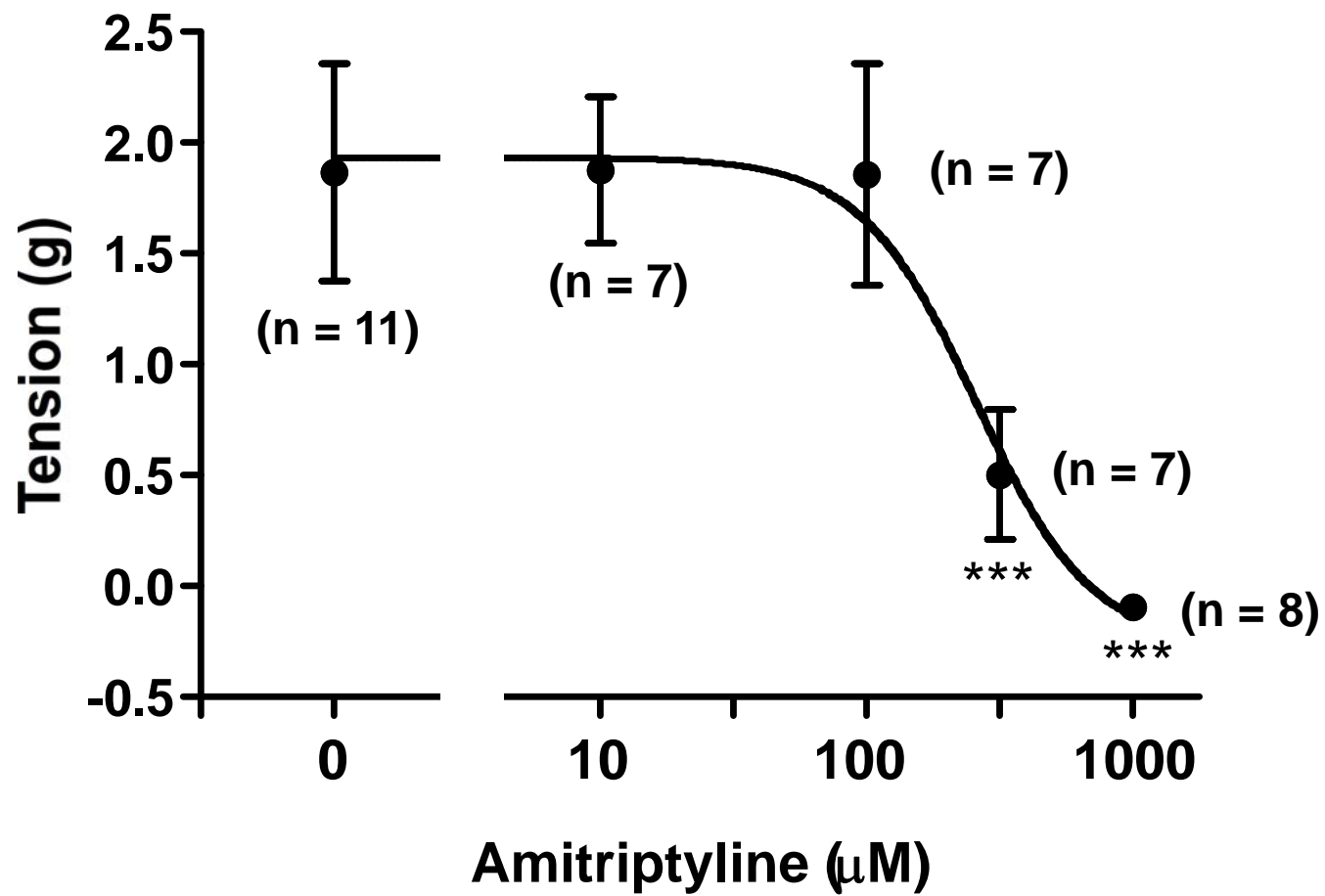


Figure 4

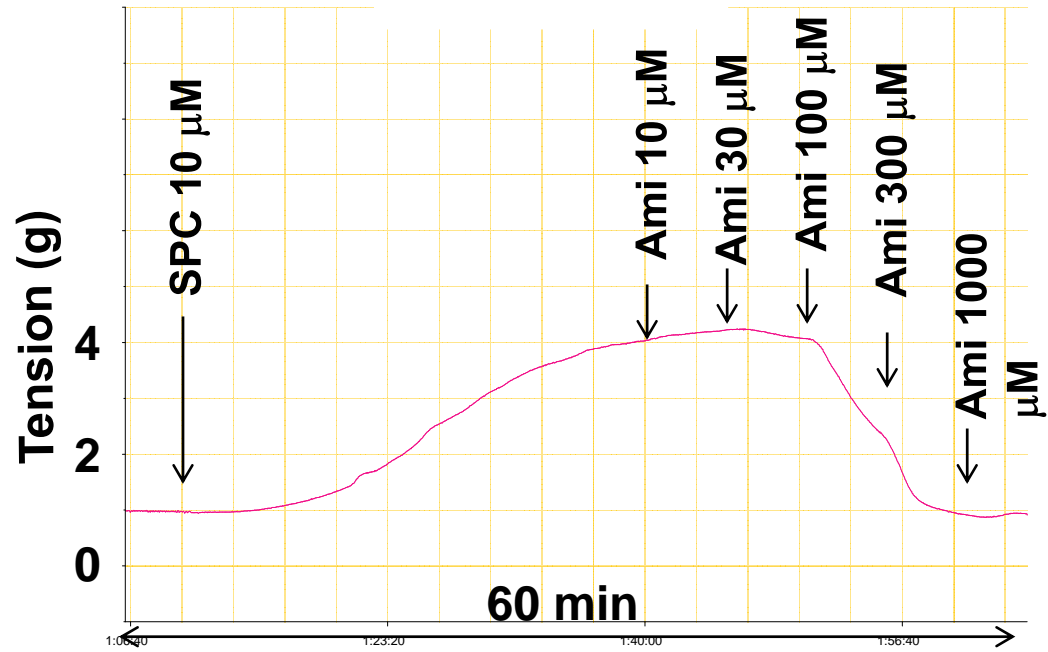


Figure 5A

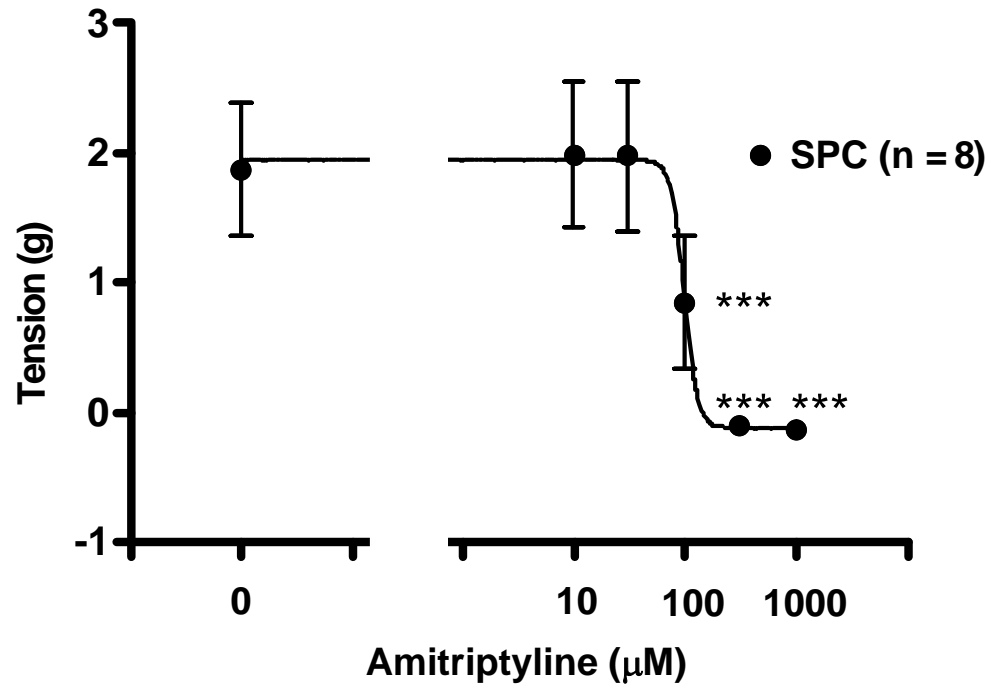


Figure 5B

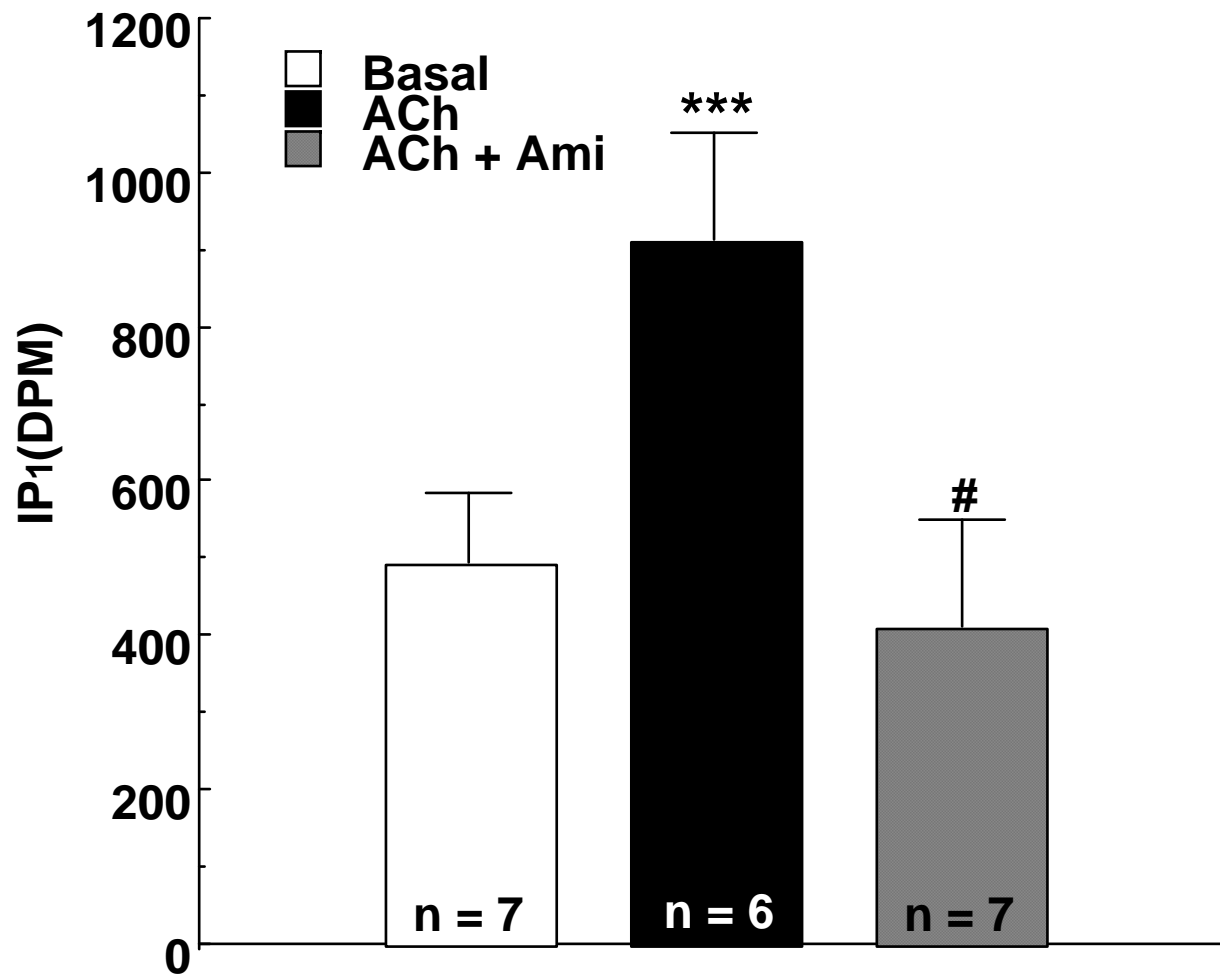


Figure 6

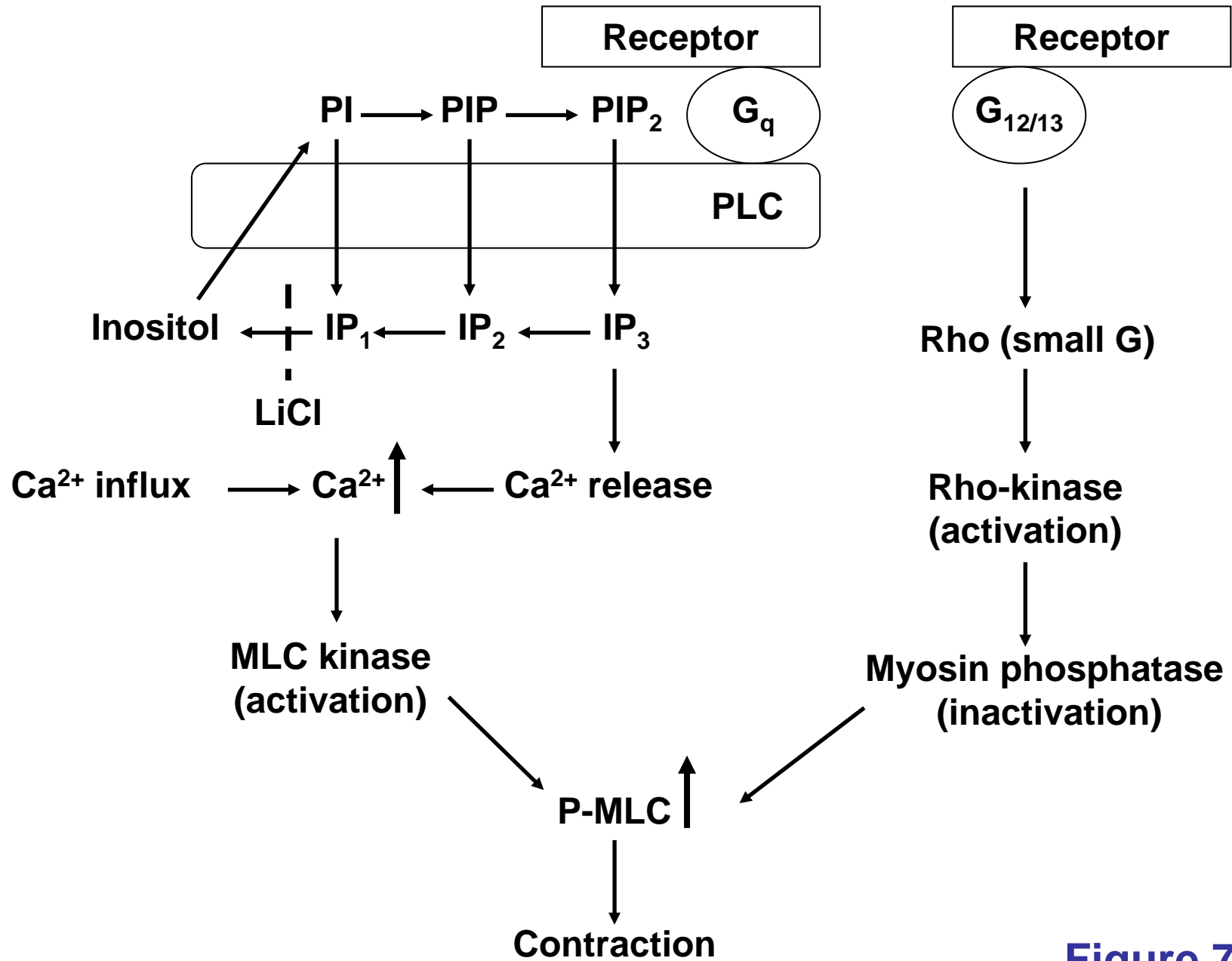


Figure 7