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Effects of amitriptyline, a tricyclic antidepressant, on smooth muscle reactivity in isolated rat trachea

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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 the contractile response to acetylcholine (ACh), electrical field stimulation (EFS), calyculin A (a myosin light chain phosphatase inhibitor), and sphingosylphosphorylcholine (SPC; a Rho-kinase activator). In addition, inositol monophosphate (IP_1) accumulation was measured to examine its effects on inositol 1, 4, 5-trisphosphate (IP_3) production during stimulation with ACh.
Results. Amitriptyline inhibited the contractile responses to ACh, EFS, calyculin A, and SPC, with the concentrations of amitriptyline (mean ± SD) required to exert 50% inhibition (IC_{50}) being 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 IP_1 accumulation.
Conclusion. The results suggest that amitriptyline does not influence tracheal smooth muscle reactivity at clinical concentrations (<1 μM), but attenuates the reactivity at supraclinical concentrations (≥1 μM). The attenuated response to ACh brought about by amitriptyline is presumably due, at least in part, to the inhibition of phosphatidylinositol (PI) metabolism. The ability of amitriptyline to inhibit the calyculin A-induced contraction suggests that amitriptyline also inhibits the Ca^{2+}-calmodulin-myosin light chain pathway independently of the inhibition of PI metabolism. Finally, the difference between the IC_{50} values for SPC-induced contraction and those for calyculin A-induced contraction suggests that amitriptyline may also inhibit the Rho-kinase pathway.

Key words Rats · Airway smooth muscle · Contractile response · Amitriptyline · Rho-kinase pathway · Phosphatidylinositol response

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Introduction

Amitriptyline, a tricyclic antidepressant, is commonly prescribed for patients with depression who undergo 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 the neural reuptake of noradrenaline, serotonin, and adenosine; blockade of α-adrenergic, cholinergic, and *N*-methyl-D-aspartate receptors; modulation of opioid receptor-mediated function; and inhibition of ion channel activities [3,4]. Thus, it would be important for anesthesiologists to understand the multiple pharmacological actions of amitriptyline 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 clinical status, as well as reducing their medication requirements [5,6]. In an 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 airway smooth muscle and autonomic nerves [7]. Specifically, activation of the postjunctional M_3 muscarinic receptor leads to airway constriction, while activation of the prejuctional M_2 muscarinic receptor results in airway relaxation. In addition, activation of the prejunctional and/or postjunctional β_2-adrenergic receptors could lead to airway relaxation. Thus, ACh (a parasympathetic neurotrans-

mitter) and noradrenaline (a sympathetic neurotransmitter) would both 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 the contractile response to both exogenously applied and endogenously released (in response to electrical field stimulation [EFS]) ACh, and found that amitriptyline attenuated the contractile response to both exogenously applied and endogenously released ACh. Therefore, we next attempted to clarify the mechanisms behind the amitriptyline-induced airway relaxation, utilizing various pharmacological tools, including guanethidine (a sympathetic suppressor), calyculin A (a myosin light chain [MLC] phosphatase inhibitor), and sphingosylphosphorylcholine (SPC; a Rho-kinase activator). Specifically, guanethidine, calyculin A, and SPC were used to examine the possible involvement of the noradrenaline-reuptake mechanism, the Ca^{2+}-calmodulin-myosin light chain kinase (MLCK) pathway, and the Rho-kinase pathway, respectively, in the amitriptyline-induced airway relaxation. In addition, inositol monophosphate (IP_1) accumulation was measured to examine whether amitriptyline attenuated the contractile response to ACh by inhibiting 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 $mg \cdot kg^{-1}$, 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, Na_2-ethylene diamine tetraacetic acid [EDTA] 0.05). The solution was continuously aerated with O_2 95%/CO_2 5% at 37°C.

Isometric tensions were measured using an isometric transducer 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 an SEN-7203 stimulator (Nihon Kohden, Tokyo, Japan) and applied between two platinum electrodes. Pulses of 2 ms and 50 V were delivered at a frequency of 25 Hz for 10 s, and a 15-min recovery period was allowed between successive trains.

Measurement of IP_1 accumulation

A technique modified from that of Brown et al. [8] was used. Inositol 1, 4, 5-trisphosphate (IP_3) is rapidly degraded into inositol monophosphate (IP_1) and subsequently recycled back to PI via free inositol. Lithium inhibits the conversion of IP_1 to inositol, and in the presence of Li^+ the accumulation rate of IP_1 reflects the extent of the PI response. We measured [^{3}H] IP_1 in tracheal slices incubated with [^{3}H] *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 O_2 95%/CO_2 5%. An aliquot of 0.5 µCi [^{3}H] *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 O_2 95%/CO_2 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; 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 [^{3}H] IP_1 was separated from [^{3}H] *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. [^{3}H] IP_1 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 the contractile response to exogenously applied ACh. Specifically, amitriptyline (1 µM-1 mM) was cumulatively applied to the rings precontracted with ACh (10 µM).

In the second series of experiments, we attempted to examine the effects of amitriptyline on the contractile

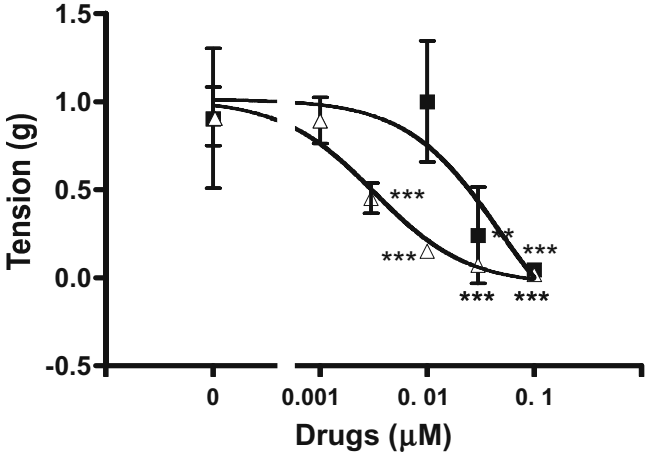

Fig. 1. The effects of tetrodotoxin (TTX; *closed squares*) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; *open triangles*) on electrical field stimulation (EFS [25 Hz])-induced contraction of rat tracheal rings (mean ± SD). Twelve tracheal rings ($n = 12$) from six rats ($N = 6$) were used in the experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ vs drugs 0

response to endogenously released ACh, utilizing the contractile response to EFS. In preliminary experiments, both tetrodotoxin (TTX; an Na channel blocker) [9] and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; an M_3-cholinergic receptor antagonist) [10] inhibited the EFS response, with values for the concentrations required to exert 50% inhibition (IC_{50}) of 20 ± 8 nM and 3.0 ± 0.8 nM, respectively; these agents eliminated the EFS response at 100 nM and 30 nM, respectively (Fig. 1). However, prazosin (an α_1-adrenergic blocker [11]), at 0.1–10 μM, and capsaicin (a neuronal desensitizer depleting calcitonin gene related peptide [CGRP] from the nerve terminals [12]), at 0.1–10 μM, did not influence the EFS response ($P > 0.05$, $n = 6$, data 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 the contractile response to ACh or EFS. Therefore, 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 the possibility that amitriptyline causes tracheal relaxation by inhibiting the reuptake of noradrenaline into the sympathetic nerves, we examined the effects of amitriptyline (1–100 μM) on the 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 the possibility that amitriptyline causes tracheal relaxation by inhibiting the Ca^{2+}-calmodulin-MLCK pathway, we examined the effects of amitriptyline on the contractile response to calyculin A (an 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 decreased significantly with time in the preliminary study.

In the fifth series of experiments, in order to investigate the possibility that amitriptyline causes tracheal relaxation by inhibiting the Rho-kinase pathway, we examined the effects of amitriptyline on the 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 method modified from Brown et al. [8] detailed above, we examined the effects of amitriptyline on ACh-indued 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 means ± SD. Concentration-effect curves were fitted by nonlinear regression (GraphPad Prism; GraphPad, San Diego, CA, USA). The results for the guanethidine data were analyzed by two-way analysis of variance (ANOVA). The interaction was statistically significant, and therefore differences between the control and guanethidine groups 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 ($n = 6$; Fig. 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 ($n = 9$; Fig. 3). No significant difference was observed between the IC_{50} value for inhibition of the ACh response and that for inhibition of the 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 the contractile response to EFS (Fig. 3). The IC_{50}

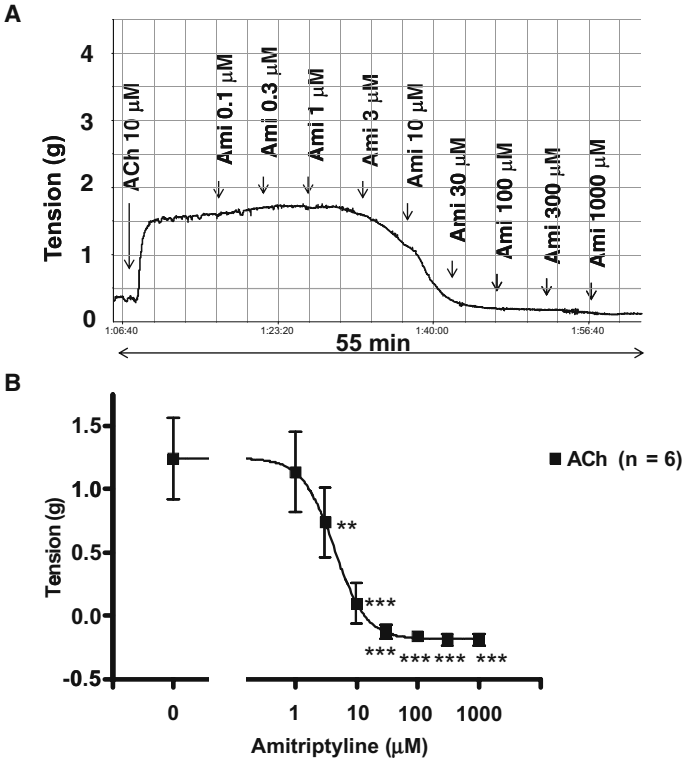

Fig. 2. **A** A recording of the effects of amitriptyline (*Ami*) on acetylcholine (*ACh*)-induced contraction of rat tracheal rings. ACh, 10 μM was added, and 10 min later, tension was measured by stepwise cumulative additions of amitriptyline. **B** The effects of amitriptyline on ACh-induced contraction of rat tracheal rings (mean ± SD, $n = 6$; number of rats, $N = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$ vs amitriptyline 0. The concentration of amitriptyline required to exert 50% inhibition (IC_{50}) of the ACh-induced contractions was 4.3 ± 1.3 μM

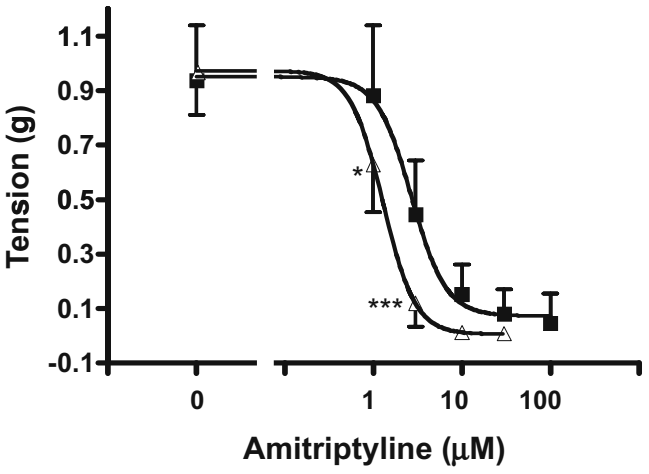

Fig. 3. The effects of amitriptyline on electrical field stimulation (EFS)-induced contraction of rat tracheal rings in the absence (control; *closed squares*) or presence of guanethidine (*open triangles*; mean ± SD, $n = 9$; number of rats, $N = 10$). $^{*}P < 0.05$; $^{***}P < 0.001$ vs control. EFS, 25 Hz; guanethidine, 10 μM. The concentrations of amitriptyline required to exert 50% inhibition (IC_{50}) of the EFS-induced contractions were 3.2 ± 1.6 and 1.3 ± 0.4 μM in the absence and presence of guanethidine, respectively

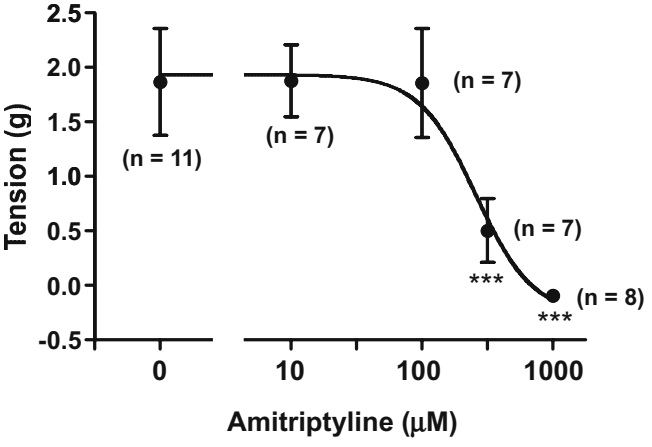

Fig. 4. The effects of amitriptyline on calyculin A (myosin phosphatase inhibitor)-induced contraction of rat tracheal rings (mean ± SD, $n = 7$–11; number of rats, $N = 15$). Amitriptyline was added prior to calyculin A, because the contraction induced by calyculin A reached maximum within 30 min, followed by spontaneous decrease to the baseline, in the preliminary study. $^{***}P < 0.001$ vs amitriptyline 0. Calyculin A, 1 μM. The concentration of amitriptyline required to exert IC_{50} of the calyculin A-induced contractions was 256.4 ± 106.4 μM

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 that 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 ± 106.4 μM ($n = 7$–11; Fig. 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 ($n = 8$; Fig. 5B), and almost eliminated it at 300 μM.

The basal IP_1 accumulation, in which no drugs were administered, was 494 ± 91 disintegration per minute (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 (Fig. 6).

Discussion

The present results indicate that amitriptyline attenuates the 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.

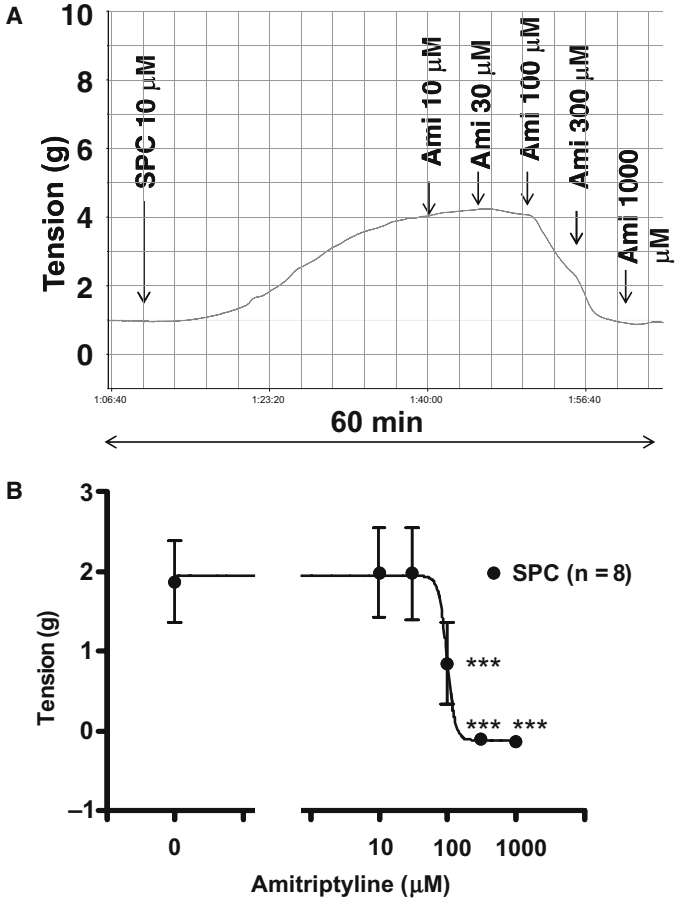

Fig. 5. **A** A recording of the effects of amitriptyline on 10-μM sphingosylphosphorylcholine (*SPC*)-induced contraction of rat tracheal rings. SPC, 10 μM was added; 30 min later, tension was measured by stepwise cumulative additions of amitriptyline. SPC-induced contractions were observed to continue for more than 90 min in the preliminary study. **B** The effects of amitriptyline on SPC (*closed circles*)-induced contraction of rat tracheal rings (mean ± SD, $n = 8$; number of rats, $N = 6$). $^{***}P < 0.001$ vs amitriptyline 0. The concentration of amitriptyline required to exert 50% inhibition (IC_{50}) of the SPC-induced contractions was 98.2 ± 21.8 μM

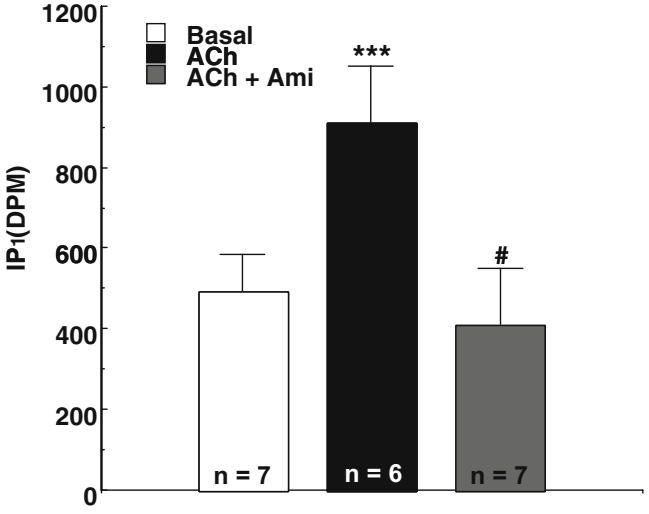

Fig. 6. The effects of amitriptyline (*Ami*, 10 μM) on inositol monophosphate (IP_1) accumulation in rat tracheal slices (mean ± SD, $n = 6$–7; number of rats, $N = 8$). *DPM*, disintegration per minute; *ACh*, acetylcholine 10 μM. $^{***}P < 0.001$ vs basal; $^{\#}P < 0.001$ vs ACh

However, the effective concentrations of amitriptyline in the present study were significantly higher than clinically relevant concentrations (<1 μM). Thus, amitriptyline would not influence the contractile response to ACh through peripheral (i.e., direct and peripheral nerve) action on airway smooth muscle in the normal clinical setting.

James et al. [16] observed the effects of tricyclic antidepressants on gastric smooth muscle contractility in guinea pigs, and found that amitriptyline reduced the contractions induced by ACh. Huang and Lau [17] reported that, in the rat isolated trachea, amitriptyline produced a shift to the right of the ACh concentration-contraction curve without affecting the maximum contraction. The IC_{50} value was 10.3 μM for inhibition of the submaximal contractile response to 1 μM ACh. This IC_{50} value is similar to the value in 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 (PIP_2) into IP_3 and diacylglycerol. IP_3 stimulates the sarcoplasmic reticulum to release Ca^{2+} that in turn activates the Ca^{2+}-calmodulin-MLCK pathway and initiates contraction (Fig. 7), while diacylglycerol activates protein kinase C, possibly leading to an increase in myofilament Ca^{2+} sensitivity. Following the initial increase in free Ca^{2+} concentration in the cytosol $[Ca^{2+}]c$ caused by the IP_3-induced Ca^{2+} release, a tonic increase in $[Ca^{2+}]c$, which is largely dependent on extracellular Ca^{2+}, is normally observed in the contractile response to receptor agonists. In previous studies with isolated human mesenteric arteries [18], human vas deferens [19], rabbit aorta [20], and guinea-pig ileum [21,22], amitriptyline inhibited the contractile response to various receptor agonists, such as noradrenaline [18–20], histamine [21], and serotonin [22]. These results suggest that amitriptyline may inhibit activation of the PI cascade and thereby attenuate smooth muscle contractile activity. Indeed, in the present study, amitriptyline inhibited the ACh-induced IP_1 accumulation, providing the first direct evidence for the amitriptyline-induced inhibition of ACh-induced IP_3 production (i.e., ACh-activation of

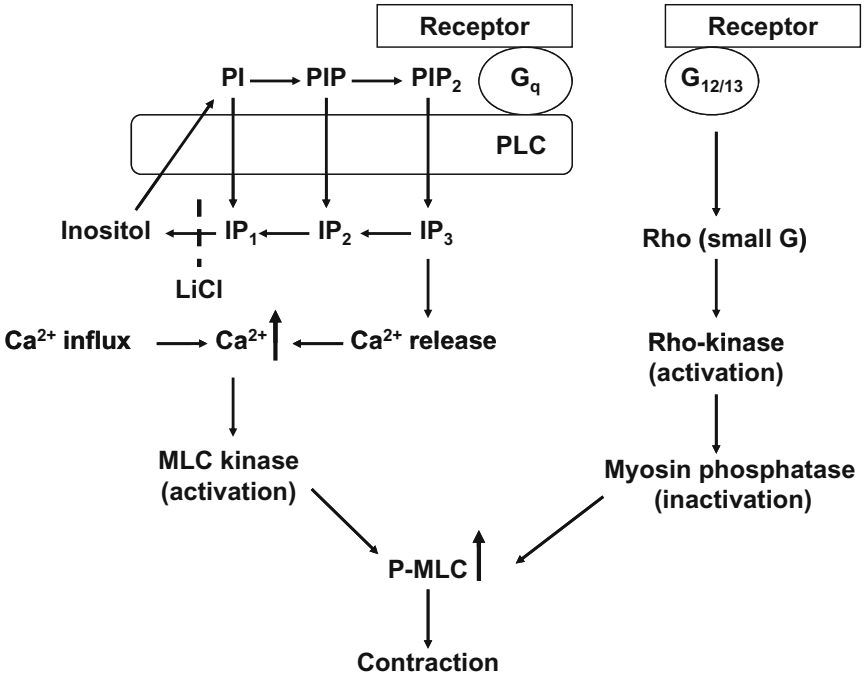

Fig. 7. Flow diagram of the phosphatidylinositol (*PI*) response and Rho-kinase pathway. *G*, G-protein; *PIP*, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4, 5-bisphosphate; IP_3, inositol 1, 4, 5 trisphosphate; IP_2, inositol bisphosphate; IP_1, inositol monophosphate; *MLC*, myosin light chain; *P-MLC*, phosphorylated myosin light chain; *PLC*, phospholipase C

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 a direct action on airway smooth muscle and/or presynaptic inhibition of the vagus nerve [23–25]. Amitriptyline inhibits the reuptake of noradrenaline into nerve terminals in the brain [3]; therefore, 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 unknown at present, and will 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 (Fig. 7). Inactivation of myosin phosphatase increases myosin light chain (MLC) phosphorylation, resulting in an increased contraction. Airway smooth muscle contraction occurs through the activation of receptors coupled with small G-proteins in canine, rabbit, and human airway smooth muscles in vitro, and involves the Rho-kinase pathway [27–30]. It has been suggested that SPC causes vasoconstriction by activating the Rho-kinase pathway in cerebral, coronary, and pulmonary arteries [15,31,32]. In the present study, amitriptyline inhibited SPC-induced contraction, suggesting the possibility that amitriptyline inhibits Rho-kinase. However, amitriptyline also inhibited the calyculin A-induced contraction, i.e., the contraction caused by the 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^{2+}-calmodulin-MLCK pathway. Nevertheless, we speculate that amitriptyline may inhibit Rho-kinase, because the IC_{50} value for inhibition of the SPC-induced contraction (98 μM) was significantly lower than that for inhibition of the calyculin A-induced contraction (256 μM). Further research—including the direct measurement of Rho-kinase activity—is required to clarify this issue.

In conclusion, amitriptyline attenuates smooth muscle reactivity at supraclinical concentrations (≥1 μM). The attenuated response to ACh brought about by amitriptyline is presumably due, at least in part, to the inhibition of PI metabolism. The ability of amitriptyline to inhibit calyculin A-induced contraction suggests that amitriptyline also inhibits the Ca^{2+}-calmodulin-MLCK pathway independently of its inhibition of PI metabolism. Finally, the difference between the IC_{50} values for the SPC-induced contraction and the calyculin A-induced contraction suggests that amitriptyline may also inhibit the Rho-kinase pathway.

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