Landiolol, a β_1-adrenoceptor antagonist, stimulates smooth muscle contraction of the rat trachea through the Rho-kinase pathway

Running Head: β_1-adrenoceptor antagonist and Rho-kinase pathway

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Abstract

Purpose The gradually progressing contraction of the airway smooth muscle is suggested to be due to the Rho-kinase signaling pathway. In our preliminary study, landiolol, a β_1-adrenoceptor antagonist, at high doses caused gradually progressing contraction, and this contraction reached a plateau after 20 min. Thus, this study was carried out to clarify whether landiolol could stimulate the Rho-kinase pathway or phosphatidylinositol (PI) response of the rat trachea. *Methods* Seventy-eight male Wistar rats weighing 250-350 g were used for the experiments. Their tracheas were cut into 3-mm-wide ring segments or 1-mm-wide slices. Measurements of isometric tension and [^{3}H] inositol monophosphate (IP_1) formed were conducted by using rat tracheal rings or slices. Data are expressed as mean ± SD, and statistical significance ($P < 0.05$) was determined using ANOVA.

Results Landiolol (700 μM)-induced contraction was completely inhibited by fasudil at 30 μM, while landiolol-induced contraction was not inhibited by 4-DAMP, ketanserin or nicardipine. Landiolol could not stimulate IP_1 accumulation.

Conclusions These results suggest that landiolol would

cause airway smooth

muscle contraction through the Rho-kinase pathway but not through PI response coupled with muscarinic M_3 receptor or 5-HT receptor or activation of L-type Ca^{++} channel.

Key words: (1) Landiolol (2) β_1-adrenoceptor antagonist (3) Rho-kinase pathway (4) Phosphatidylinositol response (5) Tracheal smooth muscle

Introduction

Among the many β_1-selective adrenoceptor antagonists used clinically, several are known to have additional β_2-effects at high doses. Landiolol, a new potent selective β_1-adrenoceptor antagonist, used in the treatment of tachyarrhythmias [1-3].

In the experiment of rat tracheal rings, contraction induced by acetylcholine (ACh) quickly progresses and reaches a plateau within 5 min, while contractions induced by anticholinesterase (anti-ChE) drugs gradually progress and reach a plateau after 30 min [4]. Although there is no significant difference in strength between the contractions induced by ACh and Anti-ChE drugs, Anti-ChE -induced contractions are completely inhibited by Rho-kinase inhibitors, while ACh-induced contraction is inhibited incompletely [4]. These suggest that gradually progressing contractions of airway smooth muscle might be responsible for the Rho-kinase pathway.

Airway smooth muscle contraction is regulated by myosin light chain (MLC) phosphorylation (Fig. 1). When receptors on the airway smooth muscle cell membranes stimulate G_q-proteins to activate phospholipase C, inositol 1,4,5 trisphosphate (IP_3) is increased. Inositol 1,4,5 trisphosphate mobilizes Ca^{++} from the sarcoplasmic reticulum, and

at the same time Ca^{++} flows inward from the extracellular space, resulting in an increase in intracellular Ca^{++} concentration. The increase in Ca^{++} activates MLC kinase, resulting in an increase in MLC phosphorylation. On the other hand, when receptors on the airway smooth muscle stimulate heterotrimeric G-proteins and subsequently Rho (small G-proteins), the Rho-kinase pathway is activated, and then myosin phosphatase is inactivated, resulting in an increase in MLC phosphorylation [5-8].

In our preliminary study, while esmolol did not affect the resting tension of rat tracheal rings, landiolol caused a gradually-progressing contraction. This contraction reached a plateau after 20 min, and then was sustained over 90 min. A submaximal dose (700 μM) of landiolol had a potency nearly equal to 10 μM acetylcholine to induce the contractile response. The mechanisms involved in the effect of landiolol to cause the contraction of airway smooth muscle are not fully understood. The present study was carried out to clarify the mechanism of action of landiolol, by examining the effects of a Rho-kinase inhibitor, a muscarinic M_3 receptor antagonist, a 5-HT receptor antagonist and an L-type Ca^{++}

channel blocker.

Materials and Methods

This study was conducted following guidelines approved by our Institutional Animal Care Committee. Seventy-eight male Wistar rats (Charles River, Yokohama, Japan) weighing 250-350 g were used for the experiments. The rats were exsanguinated under anesthesia with intraperitoneal pentobarbital (50 $mg \cdot kg^{-1}$ intraperitoneal), and the trachea was rapidly isolated.

Contractile response

Each trachea was cut into 3-mm-wide ring segments with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). We used only the distal three rings of the trachea, i.e., within 9 mm from the carina, since the contractile responses differ between the proximal and distal segments [9]. In each experiment, eight rings from three rats were used in eight organ chambers. The tracheal ring was suspended between two stainless steel hooks and placed in a 5-mL water-jacketed organ chamber (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-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

(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).

1) At time 0 landiolol was added stepwise-cumulatively to induce active contraction at 0 μM to 1000 μM in final concentrations. Eight tracheal rings from 4 rats were used in two experiments.

2) To examine whether landiolol-induced contraction could be mediated through a muscarinic receptor, a 5-HT receptor, an L-type Ca^{++} channel or Rho-kinase pathway, 4-diphenylacetoxy-N-methyl-piperidine methobromide (4-DAMP), (a muscarinic M_3 receptor antagonist), ketanserin (a 5-HT receptor antagonist), nicardipine (an L-type Ca^{++} channel blocker), fasudil (a Rho-kinase inhibitor) or none of them was added stepwise-cumulatively 30 min after the addition of landiolol, 700 μM (submaximal dose). Thirty-six tracheal rings from 18 rats were used in six experiments.

3) To examine whether an appropriate concentration of inhibitor could inhibit or reduce tracheal contraction induced by cumulative addition

of landiolol, 10 μM in final concentration each 4-DAMP, ketanserin, nicardipine, fasudil or none of them was added 15 min before the addition of landiolol. Landiolol was added stepwise-cumulatively to induce active contraction at 0 μM to 1000 μM in final concentrations. Forty tracheal rings from 18 rats were used in seven experiments.

4) To examine whether landiolol-induced contraction could be attenuated by β-receptor agonists or phosphodiesterase inhibitors, isoproterenol, salbutamol (a β_2-receptor agonist), dobutamne (a β_1-receptor agonist) or aminophylline was added stepwise-cumulatively 30 min after the addition of landiolol, 700 μM. Thirty-two tracheal rings from 21 rats were used in seven experiments.

5) To examine whether ACh-induced contraction, which shows nearly equal values to 700 μM landiolol-induced contraction could be attenuated by isoproterenol, isoproterenol was added stepwise-cumulatively 10 min after the addition of 10 μM ACh. Six tracheal rings from 3 rats were used in an experiment.

PI response

The modified technique of Brown et al. [10] was used. Inositol 1,4,5-trisphosphate (IP_3) is rapidly degraded into inositol

monophosphate (IP_1) and subsequently recycled back to phosphatidylinositol (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 PI response. We measured [^{3}H]IP_1 in the 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).

1) To examine whether landiolol-induced contraction would be mediated through PI response, we measured IP_1 accumulation in the rat tracheal slices. We used 14 rats. Landiolol, 700 μM, carbachol (CCh), 10 μM or nothing was added at Time 0. To support the negative findings, a positive control was conducted with CCh which is known to increase IP_1 accumulation. 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) in its formate form. [^{3}H]IP_1 formed in the tracheal slices was counted using a liquid scintillation counter, and was presented by disintegration per minute (DPM). The scintillation counts for the blank values (no slices present) were subtracted to obtain the experimental data.

Data are expressed as mean ± SD. The results were subjected to one-way analysis of variance followed by Scheffe's F-test. A value of $P < 0.05$ was considered statistically significant.

Results

The recording of landiolol-induced contraction of a rat tracheal ring is shown in Fig. 2A. Fig. 2B shows the effects of landiolol on resting tension of rat tracheal rings. Fig. 3A shows the effects of 4-DAMP, ketanserin, fasudil, nicardipine or none of them on landiolol (submaximal dose: 700 μM)-induced tension of rat tracheal rings. Landiolol-induced contraction was not inhibited by either 4-DAMP, ketanserin or nicardipine. Landiolol-induced contraction was completely inhibited by fasudil at a dose of 30 μM. The ID_{50} value for fasudil on landiolol-induced tracheal contraction was 4.0 ± 1.8 μM. Fig. 3B shows the effects of above compounds (10 μM each) on tracheal contraction induced by cumulative addition of landiolol. These compounds except fasudil could not inhibit landiolol-induced contraction. Fig. 4 shows the effects of isoproterenol, salbutamol, aminophylline and dobutamine on landiolol (700 μM)-induced contraction of rat tracheal rings. The ID_{50} value for isoproterenol on landiolol-induced tracheal contraction was 0.70 ± 0.38 μM. Landiolol-induced contraction was inhibited by 86% by salbutamol and by 79% by aminophylline for each at 100 μM, respectively, while landiolol-induced contraction was not inhibited

by dobutamine. ACh-induced contraction, which showed nearly equal values to landiolol (700 μM)-induced contraction, was attenuated by 29% by isoproterenol (Fig. 5), while landiolol-induced contraction was completely inhibited by isoproterenol at a dose of 10 μM (Fig. 4). The effects of landiolol and CCh on the IP_1 accumulation in rat tracheal slices are shown in Fig. 6. Carbachol but not landiolol could increase IP_1 accumulation.

Discussion

The present results show that landiolol induces contraction of the rat trachea, and that this contraction is abolished by fasudil completely, but not inhibited by 4-DAMP, ketanserin or nicardipine. The results also show that landiolol does not stimulate PI response, while it induces the contraction of rat trachea.

When agonists stimulate receptors on airway smooth muscle cell membranes, G_q- and heterotrimeric G-proteins activate the PI response and the Rho-kinase pathway, respectively, resulting in airway smooth muscle contractions (Fig. 1). Possible mechanisms involved in the landiolol-induced contraction are as follows. First, landiolol may stimulate smooth muscle contraction of the rat trachea through the Rho-kinase pathway. 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 the Rho-kinase pathway [5-8]. Rho, the small G-protein, activates Rho-kinase, which in turn inactivates myosin phosphatase. Inactivation of myosin phosphatase increases myosin light chain phosphorylation, resulting in an increased

contraction. In the present study, we examined the role of the Rho-kinase pathway in the effects of landiolol, and found that fasudil completely abolished landiolol-induced contraction of the rat trachea. Thus, it is possible that landiolol activates the upstream axis regarding Rho kinase activation including $G_{12/13}$ receptor and Rho A as well as Rho kinase itself. Second, landiolol may stimulate ACh release from postganglionic parasympathetic nerve endi ngs, which in turn activate receptor-coupled PI response, resulting in the airway smooth muscle contraction. Janssen et al. [11] examined prejunctional β-adrenoceptors in canine bronchi by observing the effects of β-receptor antagonists on field stimulation-induced contractions, and they concluded that catecholamines act on prejunctional β -receptors resulting in inhibition of cholinergic neurotransmission in canine bronchi. Landiolol may inhibit prejunctional β-receptors, resulting in activation of cholinergic neurotransmission, and subsequent stimulation of the contractile response in the rat trachea. However, in the present study, landiolol-induced contraction was not inhibited by 4-DAMP, a muscarinic M_3 receptor antagonist. Thus, landiolol-induced contraction would not be mediated by ACh.

ACh-induced contraction, which showed nearly equal values to landiolol-induced contraction, was attenuated by 29% by isoproterenol, while landiolol-induced contraction was inhibited completely by isoproterenol. Oguma et al. [12] measured tension and intracellular Ca^{++} in guinea-pig tracheal smooth muscles stimulated by methacholine, and found that in isoproterenol-induced relaxation, the reduction in tension was greater than that in intracellular Ca^{++}. They concluded that β-adrenergic action would antagonize not only Ca^{++} mobilization but also Ca^{++} sensitization. Ca^{++} mobilization is mediated through PI response, while Ca^{++} sensitization would be mediated through Rho-kinase signaling. ACh-induced contraction is mediated through both PI response and Rho-kinase signaling, while landiolol-induced contraction would be mediated only through Rho-kinase signaling. Thus, this might be a reason why landiolol-induced contraction was completely inhibited by isoproterenol.

In the present study, the effective concentration of landiolol for inducing contraction of rat tracheal rings was 300 μM ($P <$ 0.01). The peak serum concentration of landiolol is approximately 1000 ng/mL (1.8 μM) in clinical

settings(13). The concentrations used in the present study are two orders of magnitude higher than clinically relevant concentrations. Even lower concentrations of landiolol applied intravenously would distribute easily to the tracheal tissues through the circulation in clinical settings, while higher concentrations of landiolol would be needed to cross the mucous membrane or connective tissues served as a permeability barrier to reach smooth muscle in isolated tracheal tissues. However, the differences of landiolol concentrations between the present study and clinical settings are too extensive. Thus, our results suggest that landiolol would not induce bronchoconstriction in clinical settings.

Landiolol did, but esmolol did not have effects on tracheal tension. Although esmolol and landiolol are very similar pharmaceutically and pharmacologically, their action to trachea is different. Esmolol is a racemic mixture, while landiolol is optical isomer. This difference may be the difference in their action to trachea.

In conclusion, landiolol would cause airway smooth muscle contraction through activation of the Rho-kinase pathway.

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Legends

Fig. 1.

A flow diagram of the phosphatidylinositol (PI) response and Rho-kinase pathway. G: G-protein, PI: phosphatidylinositol, 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.

Fig. 2A.

A recording of landiolol-induced contraction of the rat tracheal ring.

Fig. 2B.

The effects of landiolol on the resting tension of the rat tracheal rings (mean ± SD). ** $P < 0.01$, *** $P < 0.001$ vs Landiolol 0.

Fig. 3A.

The effects of 4-DAMP (a muscarinic M_3 receptor antagonist), ketanserin (a 5-TH receptor antagonist), fasudil (a Rho-kinase inhibitor) and nicardipine (an L-type Ca^{++} channel blocker) or none of them on landiolol-induced tension of rat tracheal rings (mean ± SD). Landiolol: 700 μM. *** $P < 0.001$ vs Fasudil 0.

Fig. 3B.

The effects of 4-DAMP (a muscarinic M_3 receptor antagonist), ketanserin (a 5-TH receptor antagonist), fasudil (a Rho-kinase inhibitor), nicardipine (an L-type Ca^{++} channel blocker) or none of them on stepwise-cumulative addition of landiolol (mean ± SD). All drugs: 10 μM. * $P < 0.05$, *** $P < 0.001$ vs None.

Fig. 4.

The effects of isoproterenol (a β-receptor agonist), salbutamol (a β_2-receptor agonist), dobutamine (a β_1-receptor agonist), and aminophylline (a phosphodiesterase inhibitor) on landiolol-induced contraction of rat tracheal rings (mean ± SD). Landiolol: 700 μM. * $P < 0.05$, *** $P < 0.001$ vs Drugs 0.

Fig. 5.

The effects of isoproterenol on ACh-induced contraction of rat tracheal rings (mean ± SD). ACh: 10 μM. *** $P < 0.001$ vs Isoproterenol 0.

Fig. 6.

The effects of landiolol on IP_1 accumulation in rat tracheal slices (mean ± SD). IP_1: inositol monophosphate. DPM: disintegration per minute. Landiolol: 700 μM, CCh (carbachol): 10 μM. *** $P < 0.001$ vs basal or landiolol.

Airway smooth muscle contraction

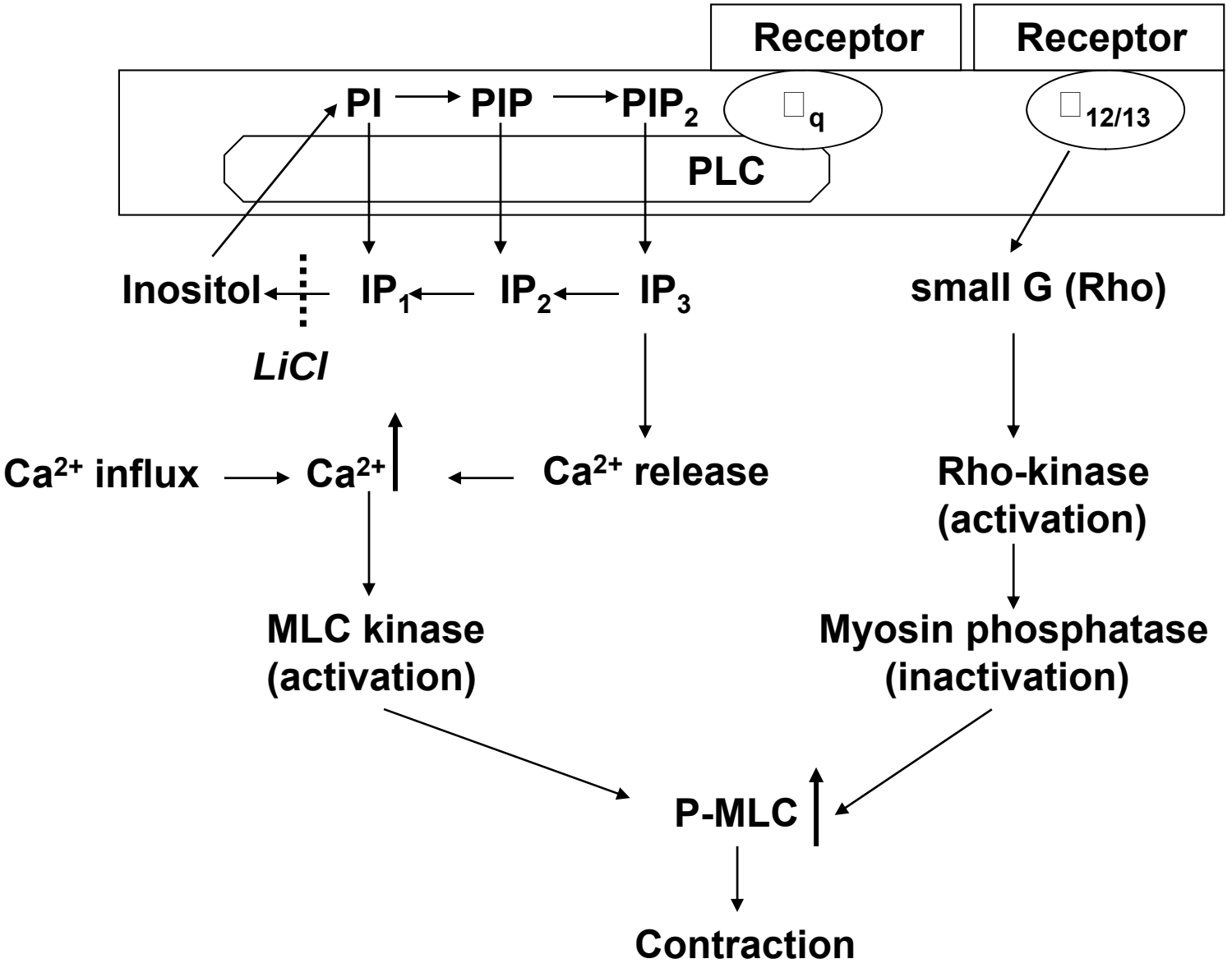


Figure 1

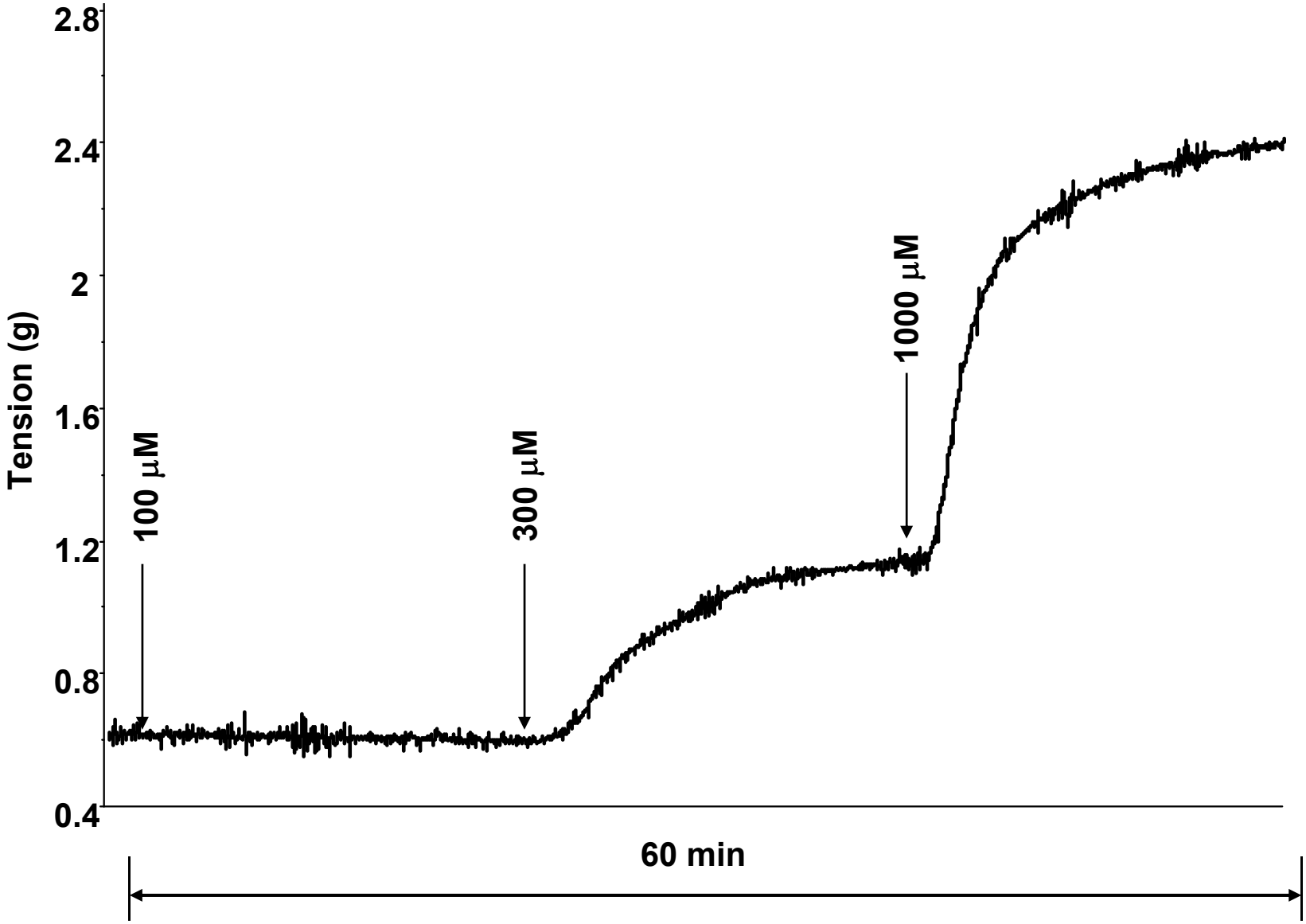


Figure 2A

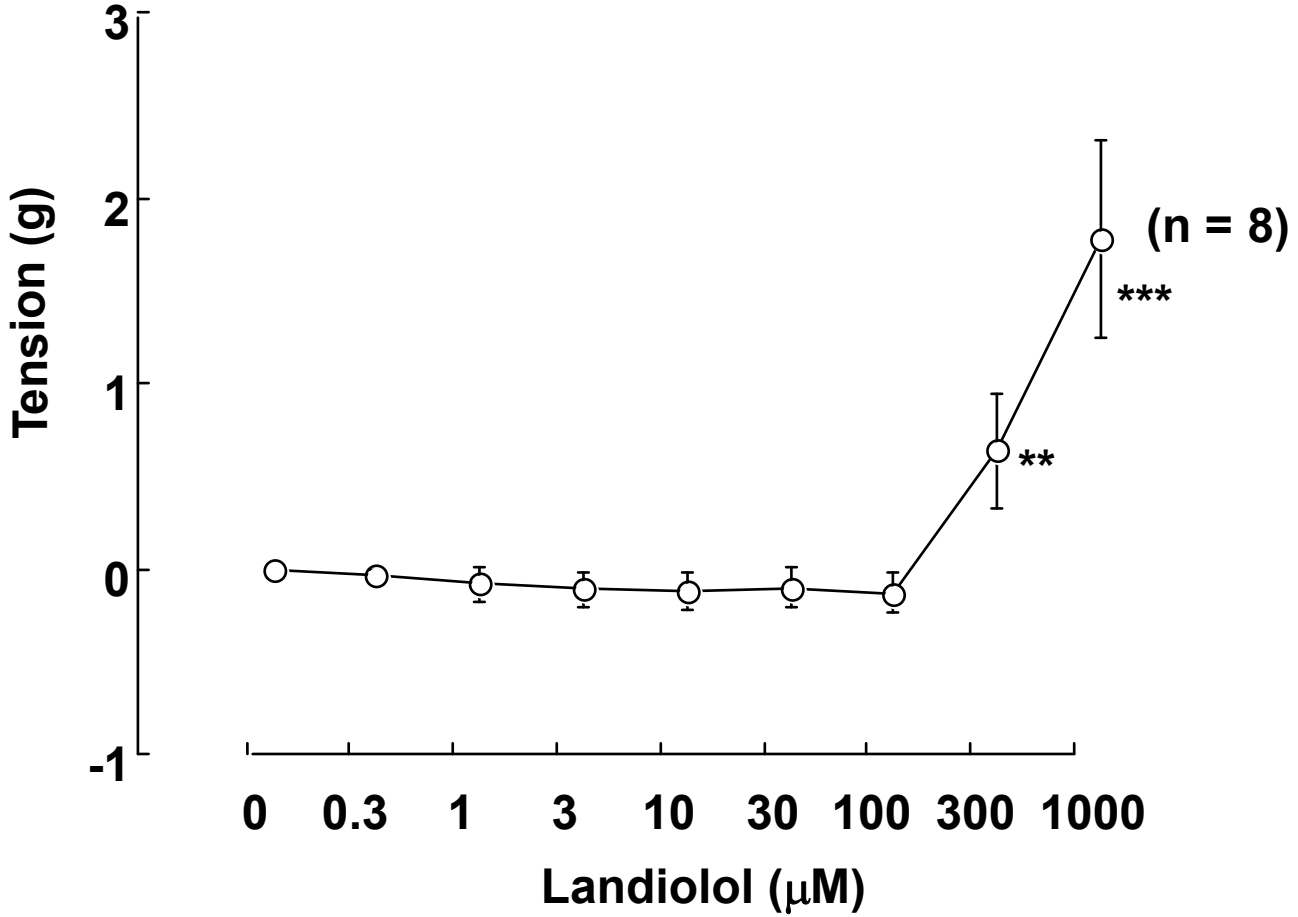
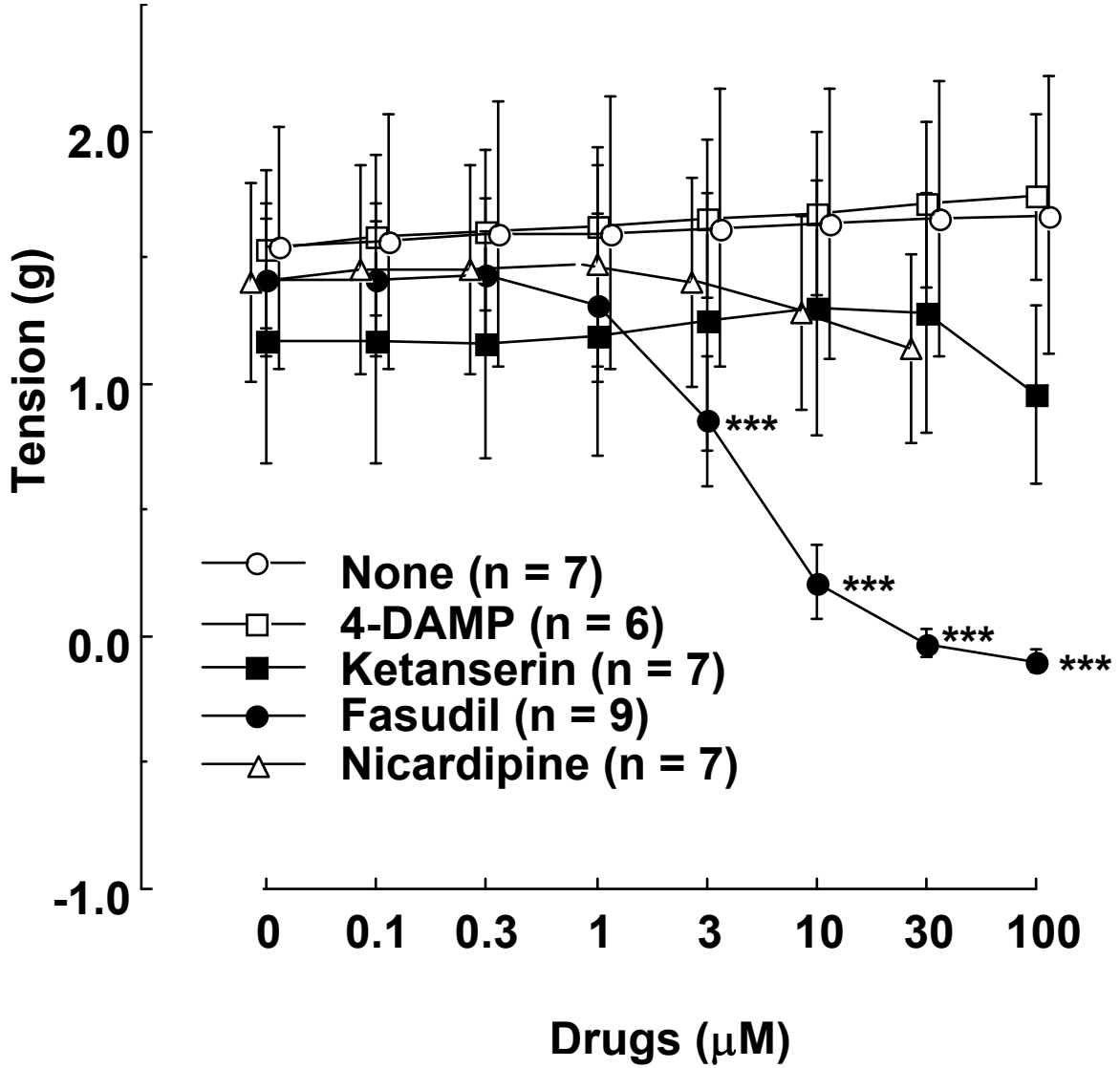


Figure 2B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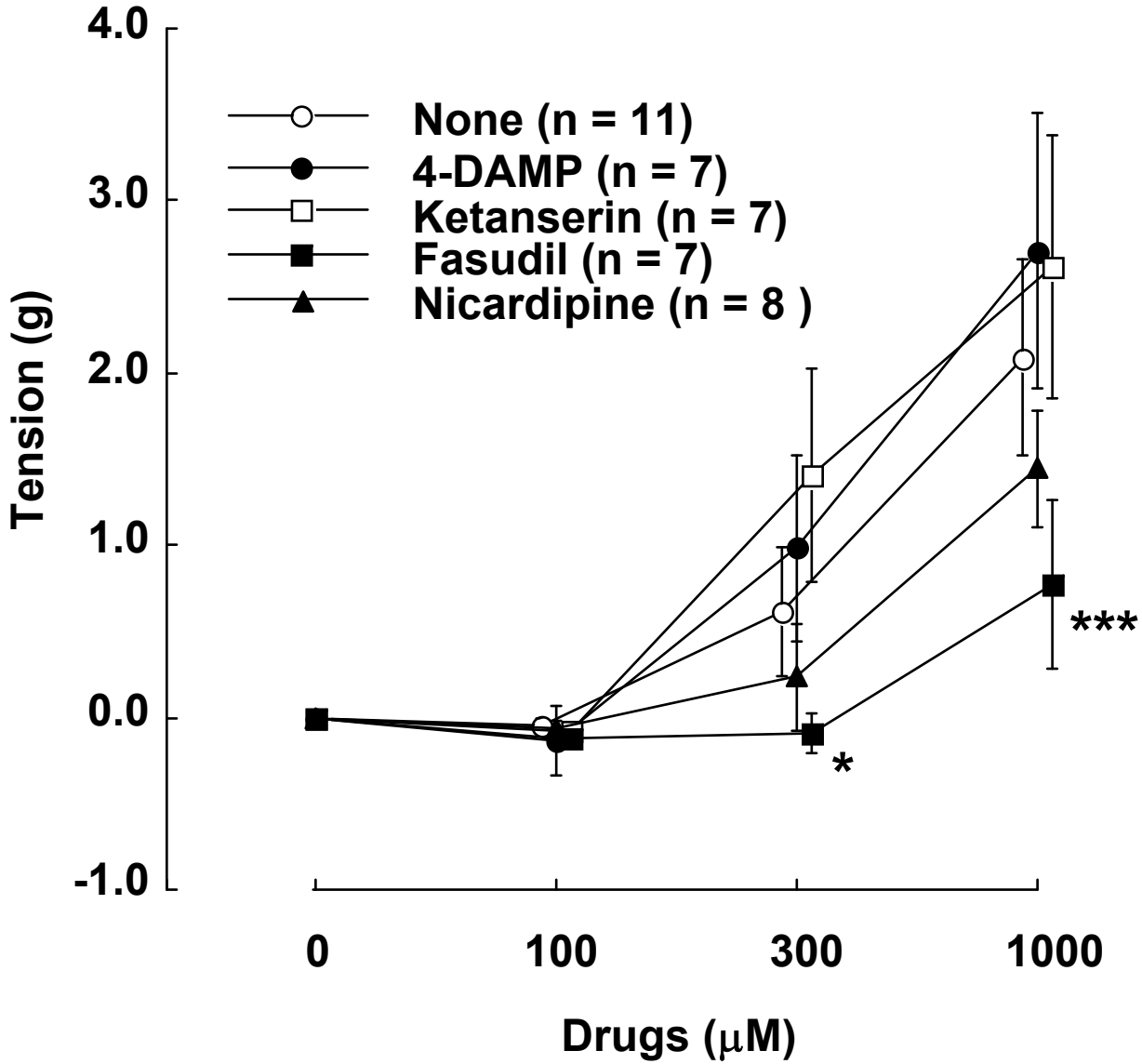
1.0
0.0
-1.0
Tension (g)
None (n = 7)
4-DAMP (n = 6)
Ketanserin (n = 7)
Fasudil (n = 9)
Nicardipine (n = 7)
***
***
***
***
0
0.1
0.3
1
3
10
30
100
Drugs (μM)

Figure 3A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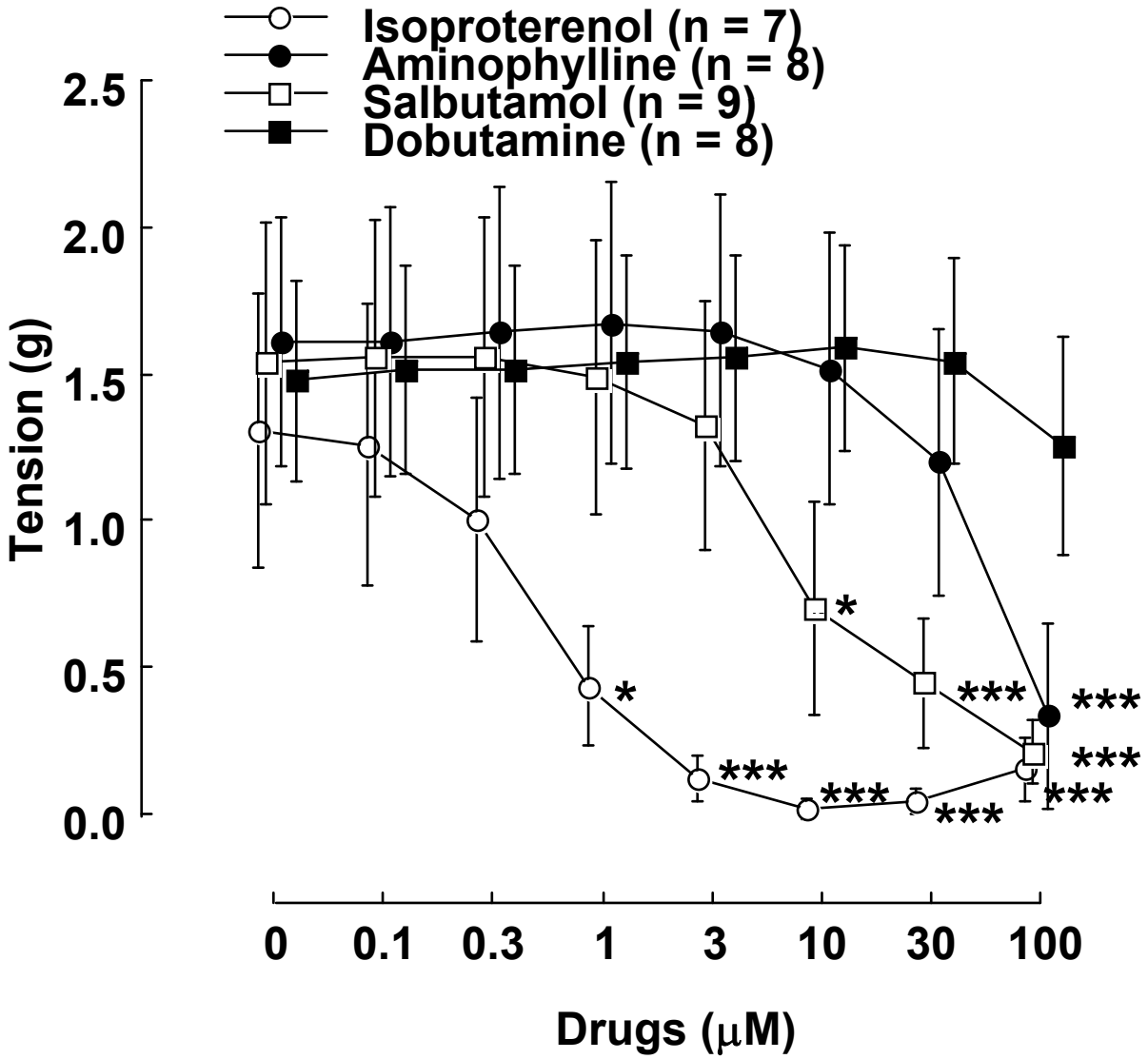
3.0
2.0
1.0
0.0
-1.0
Tension (g)
None (n = 11)
4-DAMP (n = 7)
Ketanserin (n = 7)
Fasudil (n = 7)
Nicardipine (n = 8)
*
***
0
100
300
1000
Drugs (μM)

Figure 3B


Isoproterenol (n = 7)
Aminophylline (n = 8)
Salbutamol (n = 9)
Dobutamine (n = 8)
Tension (g)
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2.0
1.5
1.0
0.5
0.0
0
0.1
0.3
1
3
10
30
100
Drugs (μM)
*
***

Figure 4

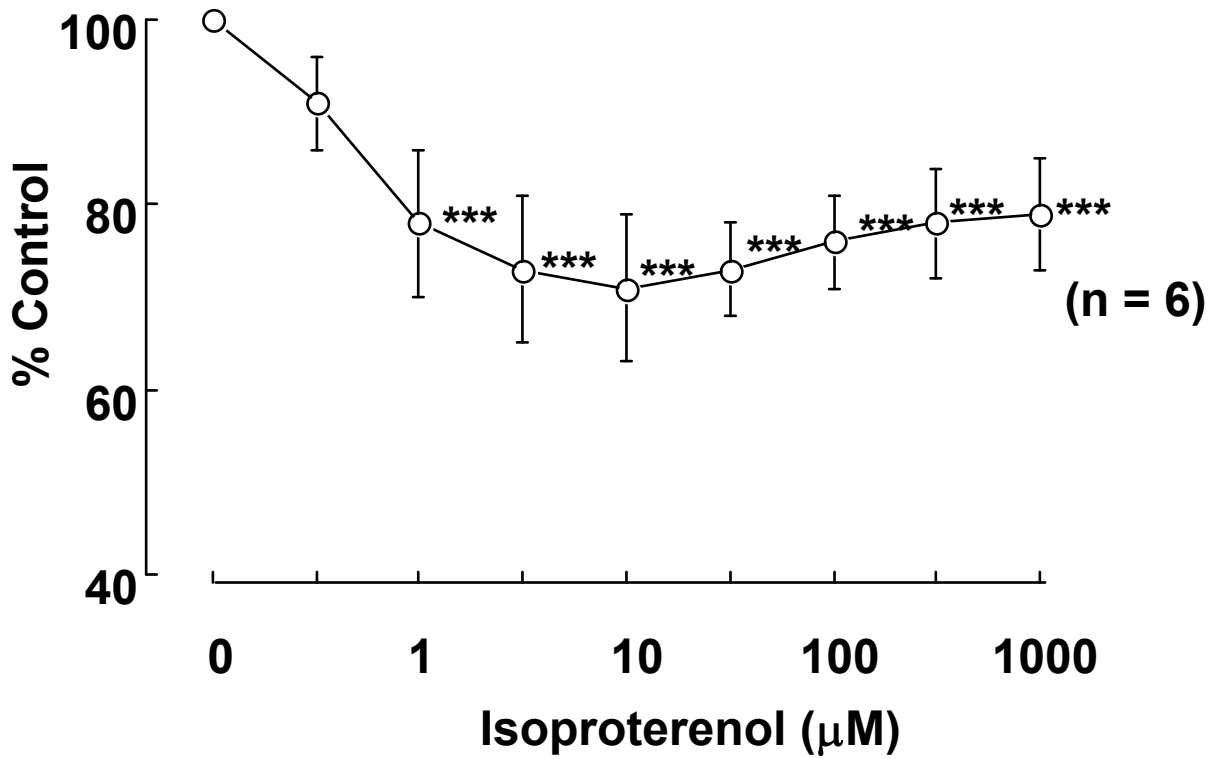

Figure 5

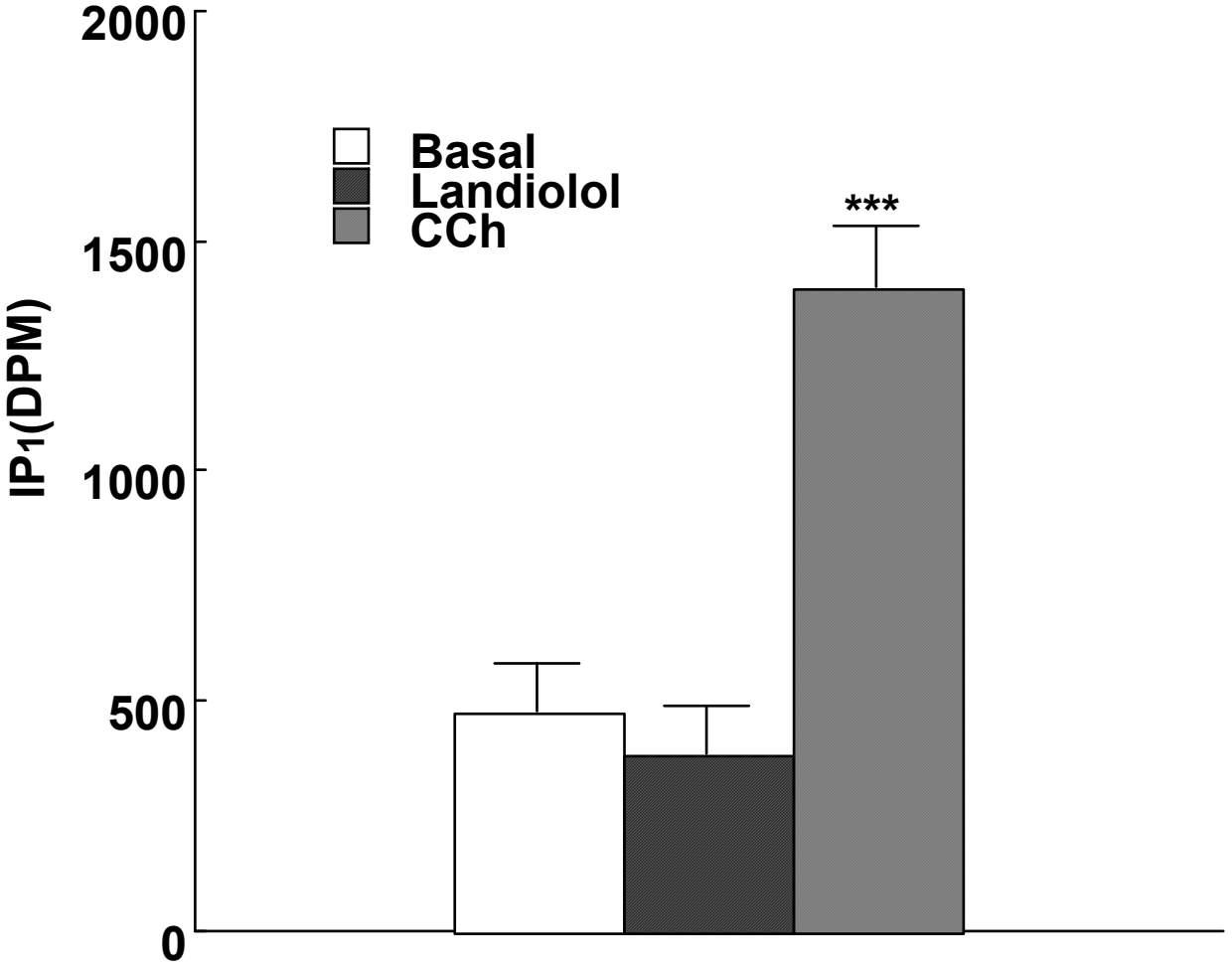

Figure 6