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Pharmacology

Imidazole-induced contractions in bovine tracheal smooth muscle are not dependent on the cAMP pathway

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ABSTRACT. The mechanism of imidazole-induced contraction on the bovine tracheal smooth muscle was investigated. Imidazole induced muscle contraction in a concentration-dependent manner on bovine, porcine and guinea-pig tracheas, but not in rat or mouse. In bovine tracheas, imidazole was cumulatively applied and induced muscle tension and increasesd intracellular Ca^{2+} level in a concentration -dependent manner. Imidazole, even at 300 μM, the concentration at which maximum contractile response occurs, did not significantly increase in cAMP content relative to control. Atropine inhibited imidazole-induced contraction at a concentration-dependent manner and pretreatment of hemicholinium-3 almost abolished imidazole-induced contraction. Conversely, pretreatment of tripelennamine, indomethacin or tetrodotoxin did not affect imidazole-induced contraction. Acetylcholine or eserine induced contraction in bovine, porcine, guinea pig, rat and mice trachea in a concentration-dependent manner. However, there was little difference in the rank order of maximum contraction of these agents. Imidazole-induced contraction was greater in bovine trachea compared to the other species tested. Further, cAMP did not appear to play a role in imidazole-induced contraction, suggesting other mechanisms, such as the release of endogenous acetylcholine.

KEY WORDS: cAMP, imidazole, smooth muscle, trachea

Cyclic adenosine monophosphate (cAMP), as well as cyclic guanosine monophosphate (cGMP), is important second messenger and closely involved in development of diseases. Many drugs targeting cAMP have been launched to treat for cardiovascular and respiratory diseases. cAMP is synthesized by adenylyl cyclase (AC) stimulating with β adrenergic agonist, and degraded by phosphodiesterase (PDE). Therefore, β adrenergic agonists, AC activators, PDE inhibitors and membrane permeable cAMP are used as therapeutic agents for heart failure.

Currently, PDEs are classified into 11 families [1] and numerous drug discovery studies have been done because of specific distribution on various organ [12]. On the other hand, imidazole is known as a PDE activator, but it will be not a therapeutic agent because it is predicted to accelerate the hydrolysis of cAMP, but it is important research tool for promoting AC/cAMP/PDE signaling [2].

Tracheal smooth muscle is an important research tool in the study of respiratory diseases, and bronchial smooth muscle is directly linked to disease pathogenesis [16]. However, since bronchus of rodents such as mice, is small to use research and from the concept of 3R of laboratory animal studies, porcine and bovine tracheas, which are waste organs of food animals, are useful for research. Especially, it is extremely important to study of AC/cAMP/PDE signaling in tracheal smooth muscle. It is also well known that the trachea is one of the organ, which has animal species difference for drug reaction [11, 19]. We have continued research on the agents related to signal transduction of cAMP and cGMP in bovine and porcine smooth muscle [6–9].

In this study, we compared the effect of imidazole in tracheal smooth muscle contraction of various animals and investigated the mechanism of imidazole induced muscle tension in bovine tracheal smooth muscle.

MATERIALS AND METHODS

Muscle preparations and tension measurement

Tracheas from adult bovine and porcine of either sex were obtained from a local abattoir. Male Hartley guinea-pig (300–450 g),

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Wistar rats (250–300 g) and ddy mouse (25−35 g) were anesthetized using sodium pentobarbital (50 mg/kg, i.p.) and euthanized by exsanguination. The trachea was isolated and ring strips were obtained. The epithelium was removed by gently rubbing the inner surface of the vessel with forceps. The smooth muscle was excised from the cartilage, and the epithelium and connective tissues were removed. The muscle strips were incubated with physiological salt solution (PSS) containing (in mM) 136.8 NaCl, 5.4 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 11.9 $NaHCO_3$ and 5.6 glucose. The PSS was aerated with 95% O_2 and 5% CO_2 at 37°C to adjust the pH to 7.2. High K^+ solution was made by replacing NaCl with equimolar KCl. All experiments were performed in accordance with institutional guidelines for animal care at Nippon Veterinary and Life Science University.

Muscle tension was recorded isometrically. One end of each strip was bound to a glass holder and the other end was connected by silk thread to a strain-gauge transducer (TB-611T; Nihon Kohden, Tokyo, Japan) in an organ bath containing PSS with a resting tension of 5−30 mN. The muscle strips were equilibrated for 30 min to obtain a stable contractility induced by 65 mM KCl. The developed tension was expressed as a percentage by assuming the values at rest in normal PSS to be 0% and the contraction induced by 65 mM KCl at 10 min to be 100%.

Simultaneous measurement of muscle tension and intracellular ($[Ca^{2+}]_{i)}$ level

The $[Ca^{2+}]_i$ level was measured simultaneously with muscle tension as reported previously [8]. Muscle strips were incubated with PSS containing 5 μM fura2/AM for overnight at 4°C. Cremophor EL (0.02%), a noncytotoxic detergent, was also added to increase the solubility of fura 2/AM. One end of the muscle was pinned to the bottom of the organ bath that was filled with 8 m*l* of PSS, and the other end was attached to the transducer with a silk thread. The muscle strip was kept horizontally in the organ bath. The muscle strip was alternately excited using light at 340 nm and 380 nm through a rotating filter wheel, and the 500 nm emission was measured through a band-pass filter with a fluorometer (CAF-110; Japan Spectroscopic Co., Ltd., Tokyo, Japan). The F340/F380 ratio was recorded as an indicator of the amount of $[Ca^{2+}]_i$. The fluorescence ratio was expressed as a percentage by assigning the values at rest in normal PSS to be 0% and those at a steady contraction in 65 mM KCl to be 100%.

Assay of cAMP content

In the bovine trachea, cAMP content was measured by enzyme immunoassay, as reported previously [7]. The trachea was incubated with imidazole (300 μM) or vehicle for 10 min. After incubation with these agents, the aorta was rapidly frozen in liquid nitrogen and stored at –80°C until homogenized in 6% trichloroacetic acid (0.5 m*l*). The homogenate was centrifuged at 3,000 ×g for 15 min and the supernatant was washed with 2.0 m*l* of water-saturated diethylether four times; the cAMP content was assayed by using an enzyme immunoassay kit (GE Healthcare/Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). cAMP content is expressed as picomoles per gram (pmol/g) of wet weight of tissue.

Chemicals

Chemicals used were imidazole, tetrodotoxin (Wako Pure Chemical, Osaka, Japan), atropine sulfate salt monohydrate, tripelennamine hydrochloride, indomethacin, hemicholinium-3, acetylcholine chloride, eserine (Sigma-Aldrich, St. Louis, MI, U.S.A.), fura2/AM (Dojindo Laboratories, Kumamoto, Japan), and cremophor EL (Nacalai Tesque, Kyoto, Japan).

Statistics

Values are expressed as mean ± SEM and statistical analyzes were performed by Student's *t*-test and two-way analysis of variance, followed by the Bonferroni post-hoc test. Statistical significance was established at a *P*-values less than 0.05. Calculations and statistical analyses were performed using GraphPad Prism4 and Excel 2010 for Windows.

RESULTS

Imidazole-induced contraction in trachea of various animals

After confirming KCl (65 mM) induced sustained contraction, imidazole was cumulatively applied. Imidazole induced contraction in bovine, porcine and guinea pig trachea in a concentration-dependent manner, but did not affect in rat and mice (Fig. 1). Based on KCl-induced contraction, the rank order of maximum contraction was bovine>porcine>guinea-pig>rat=mouse.

Effects of imidazole on muscle tension and $[Ca^{2+}]_i$ level in bovine trachea

It has been suggested that imidazole induced muscle tension by increases Ca^{2+} influx in rat uterus [15]. Therefore, the effects of imidazole on $[Ca^{2+}]_i$ level in bovine trachea.

After confirming KCl (65 mM) induced muscle tension and increases on $[Ca^{2+}]_i$ level, imidazole was cumulatively applied. Imidazole induced muscle tension and increases on $[Ca^{2+}]_i$ level at a concentration-dependent manner in bovine trachea (Fig. 2A). Cumulative addition of KCl also induced a graded increase on $[Ca^{2+}]_i$ level and muscle tension. However, cumulative addition of imidazolel induced greater contraction than high K^+ at a given $[Ca^{2+}]_i$ level (Fig. 2B). Moreover, imidazole-induced muscle tension and increases on $[Ca^{2+}]_i$ level were completely inhibited by 1 μM atropine (Fig. 2A).

Effects of Imidazole on cAMP contents in bovine trachea

Imidazole, even at 300 μM, the concentration at which maximum contractile response occurs, did not increase significant increase in cAMP content relative to control (control [n=4]; 63.4 ± 5.0 pmol/g wet weight; imidazole [n=4]; 70.2 ± 7.2 pmol/ g wet weight).

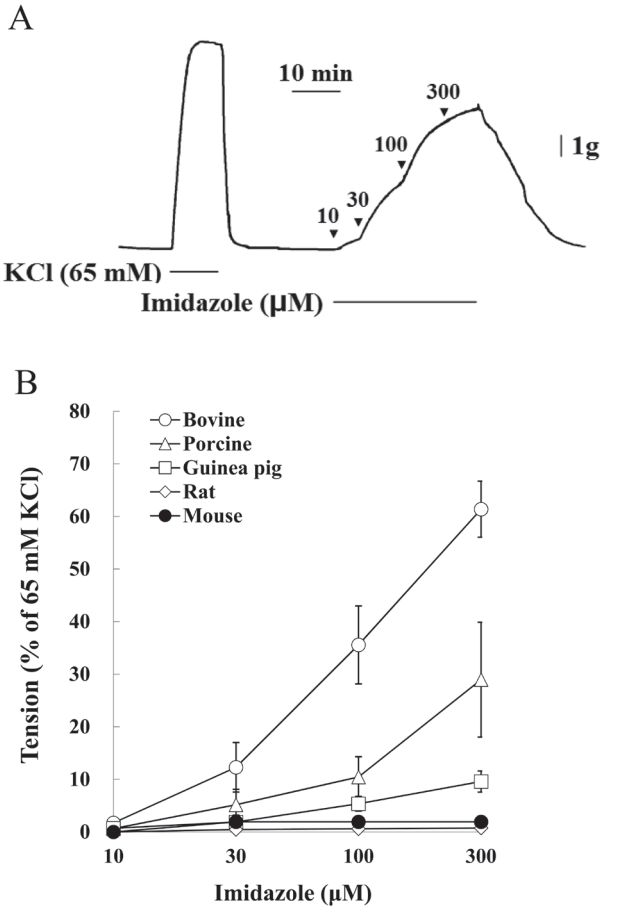

Fig. 1. Effect of imidazole on muscle tension in various species trachea. (A) A typical trace in which imidazole (10-300 μM)-induced contraction in bovine trachea, (B) imidazole-induced contraction in various species trachea. Ordinate: tension. Precontraction induced by 65 mM KCl at 10 min was considered to be 100% or resting tension was considered to be 0%. Abscissa: imidazole concentration. Each point represents the mean of 4–5 preparations. Vertical bars indicate SEM.

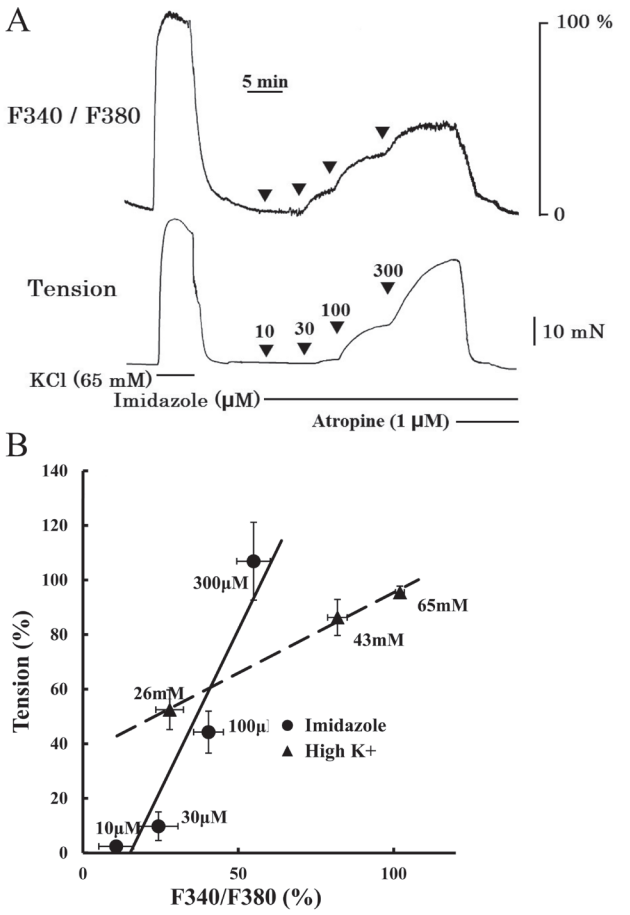

Fig. 2. Changes of imidazole induced increases in $[Ca^{2+}]_i$ level (F340/F380) and muscle tension (Tension) in bovine trachea. The $[Ca^{2+}]_i$ level and muscle tension induced by 65 mM KCl at 5 min were considered to be 100%. (A) A Typical trace of the effects of imidazole and atropine in four experiments is shown. (B) Relationship of $[Ca^{2+}]_i$ level and muscle tension induced by high K^+ or imidazole.

Effects of various inhibitors on Imidazole-induced contraction in bovine trachea

Pretreatment of bovine tracheas with atropine (1–100 nM) inhibited imidazole-induced contraction in a concentration-dependent manner (Fig. 3A).

On the other hand, pretreatment of tripelennamine (1 μM), a H_1 receptor antagonist, indomethacin (10 μM), a cyclooxygenase inhibitor, or tetrodotoxin (0.3 μg/m*l*), Na^+ channel blocker did not affect imidazole-induced contraction. However, pretreatment of hemicholinium-3 (HC-3, 1 mM), a choline reuptake blocker, almost abolished imidazole-induced contraction (Fig. 3B).

Acetylcholine- and eserine -induced contraction in trachea of various animals

After confirming KCl (65 mM) induced sustained contraction, acetylcholine (ACh) or eserine, a cholinesterase inhibitor, was cumulatively applied. ACh induced contraction in bovine, porcine, guinea pig, rat and mice trachea in a concentration -dependent manner (Fig. 4A). Based on KCl-induced contraction, the rank order of maximum contraction was bovine=porcine=rat=mouse>guinea-pig.

Eserine, also induced contraction in bovine, porcine, guinea pig, rat and mice trachea in a concentration -dependent manner (Fig. 4B). Based on KCl-induced contraction, the rank order of maximum contraction was the same with the order of ACh-induced contraction.

DISCUSSION

The present study results show that imidazole-induced contraction of tracheal smooth muscle was greater in bovine tissue than in

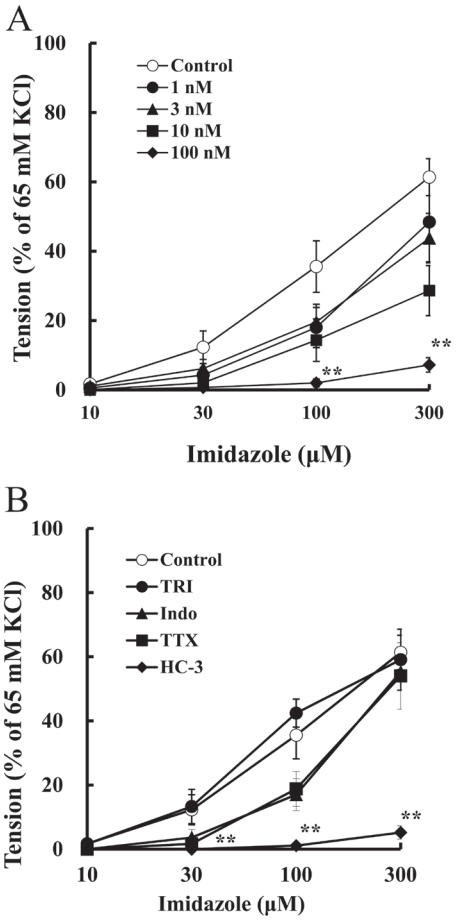

Fig. 3. Effect of atropine (1−100 nM, A) or tripelennamine (TRI, 1 μM), indomethacin (Indo, 10 μM), tetrodotoxin (TTX, 0.3 μg/m*l*) or hemicholinium-3 (HC-3, 1 mM, B) in imidazole-induced muscle tension on bovine trachea. (A, B) Ordinate: tension. Precontraction induced by 65 mM KCl at 10 min was considered to be 100% or resting tension was considered to be 0%. Abscissa: imidazole concentration. Each point represents the mean of 4–5 preparations. Vertical bars indicate SEM. **: Significant difference from each respective control, with $P<0.01$.

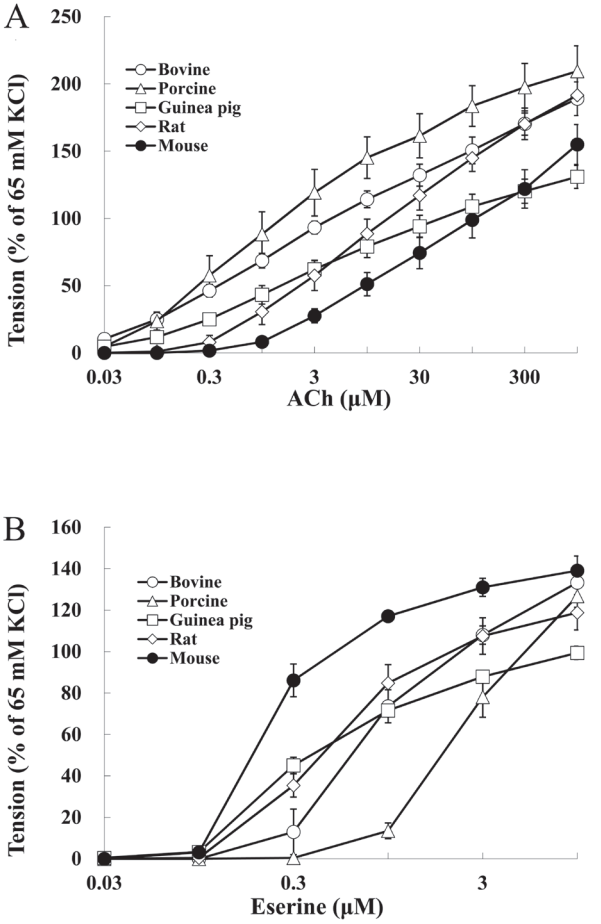

Fig. 4. Effect of acetylcholine (ACh, 0.03–1,000 μM, A) and eserine (0.03–10 μM, B) on muscle tension in various species trachea. Ordinate: tension. Precontraction induced by 65 mM KCl at 10 min was considered to be 100% or resting tension was considered to be 0%. Abscissa: ACh or eserine concentration. Each point represents the mean of 4–6 preparations. Vertical bars indicate SEM.

the other animal species tested. 1) Specifically, when we compared imidazole-induced maximum contraction based on KCl-induced contraction between species, the rank order was bovine>porcine>guinea-pig>rat=mouse. 2) In our investigation of the underlying imidazole-induced muscle tension in bovine tracheal smooth muscle, we found that (1) imidazole did not affect cAMP content, (2) atropine inhibited imidazole-induced muscle tension and increases of $[Ca^{2+}]_i$ level and (3) HC-3 almost abolished imidazole-induced muscle tension.

PDE4 inhibitors induce more effective relaxation compare to the other selective PDEs inhibitors in tracheal smooth muscle [5, 17]. Moreover, it is known that PDE4 selectively hydrolyzes cAMP [1]. Imidazole, a PDE activator [2], can enhance rhythmic contraction of the rabbit uterus by stimulating PDE [4], suggesting that cAMP may be involved in imidazole-induced muscle tension. However, in our study, imidazole did not affect cAMP contents in bovine trachea, even at 300 μM, the concentration at which maximum contractile response occurs. This result indicates that imidazole-induced contraction in bovine trachea is not due to the decreases in cAMP content by activating PDE.

It has been reported that imidazole induced muscle contraction in rat uterus by increases of Ca^{2+} influx [18]. In the current study, we also found that imidazole induced muscle contraction and increases on $[Ca^{2+}]_i$ level in a concentration-dependent manner in bovine trachea. However, cumulative addition of imidazole induced greater contraction than high K^+ at a given $[Ca^{2+}]_i$ level. Cumulative addition of carbachol induced greater contraction than high K^+ at a given $[Ca^{2+}]_i$ level in tracheal smooth muscle [15]. Additinally, in our study, atropine completely inhibited imidazole-induced muscle tension and increases on $[Ca^{2+}]_i$ level in bovine trachea. These data suggest that the imidazole-induced increases on $[Ca^{2+}]_i$ level by endogenous ACh release. Imidazole can induce contraction by augmenting the release of ACh in guinea pig ileum [10, 13]. In the present study, pretreatment with atropine or

HC-3, but not with indomethacin or TTX, inhibited imidazole-induced contraction in bovine trachea. It is well known that HC-3 is choline uptake inhibitor. Recently, it has been reported that HC-3, at low concentrations, act as inhibitors of high affinity choline transporter-1 (CHT1), at high concentrations, act as inhibitors of low affinity choline transporters [14]. In this study, we used HC-3 (1 mM) at high concentration. Differences in the efficacy of imidazole-induced contractions in various animals may be due to differences in expression of such transporters. Further research will clarify this difference.

Creese and Denborough [3] suggest that imidazole inhibited histaminase, since imidazole was able to contract guinea-pig isolated tracheal smooth muscle and also enhanced muscle tension induced by histamine, and H_1 antagonist inhibited imidazole-induced muscle tension. However, in our study, tripelennamine, a H_1 antagonist, did not affect imidazole- induced contraction in bovine trachea. These data suggest that imidazole-induced contraction in bovine trachea may be due to stimulate release of endogenous ACh.

In this study, exogenous ACh and eserine, a cholinesterase inhibitor, concentration-dependently induced contractions in bovine, porcine, guinea pig, rats and mice trachea with similar efficacy of maximum contractions. Therefore, it is suggested that the difference of imidazole-induced contraction in various animals do not involve in difference of affinity of muscarinic receptor or activity of cholinesterase.

The parasympathetic nerve regulates the symptoms and inflammation of allergic responses primarily by signaling via the peripheral muscarinic receptor.

Tracheal smooth muscle is as a classical effector of parasympathetic signaling and has been a target of research in conditions such as airway narrowing.

Isolated trachea from rodents such as mouse and rat are usually used for ex vivo experiments. However, we showed that imidazole-induced contraction in the bovine trachea was greater than that in mouse and rat, and that imidazole-induced contraction was due to stimulate release of endogenous ACh. Thus, our study demonstrates that bovine trachea may be a useful tool to investigate the asthma regulating parasympathetic nerve.

In conclusion, we showed that imidazole-induced muscle tension in bovine trachea was greater than the muscle tension in other species tested. Further, cAMP did not appear to play a role in imidazole-induced muscle tension, suggesting another mechanism is involved. It is possible that imidazole-induced muscle tension in bovine trachea may have been due to stimulate release of endogenous ACh.

REFERENCES

1. Bender, A. T. and Beavo, J. A. 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol. Rev.* **58**: 488–520. [Medline] [CrossRef]
2. Butcher, R. W. and Sutherland, E. W. 1962. Adenosine 3′,5′-phosphate in biological materials. I. Purification and properties of cyclic 3′,5′-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3′,5′-phosphate in human urine. *J. Biol. Chem.* **237**: 1244–1250. [Medline]
3. Creese, B. R. and Denborough, M. A. 1982. The effects of imidazole on contractility and cyclic AMP levels of guinea-pig tracheal smooth muscle. *Clin. Exp. Pharmacol. Physiol.* **9**: 145–155. [Medline] [CrossRef]
4. Currie, W. B. 1980. Enhanced excitability of the uterus of the pregnant rabbit by imidazole stimulation of cyclic AMP phosphodiesterase. *J. Reprod. Fertil.* **60**: 369–375. [Medline] [CrossRef]
5. Kaneda, T., Watanabe, A., Shimizu, K., Urakawa, N. and Nakajyo, S. 2005. Effects of various selective phosphodiesterase inhibitors on carbachol-induced contraction and cyclic nucleotide contents in the guinea pig gall bladder. *J. Vet. Med. Sci.* **67**: 659–665. [Medline] [CrossRef]
6. Kaneda, T., Kido, Y., Tajima, T., Urakawa, N. and Shimizu, K. 2015. PDE4 and PDE5 regulate cyclic nucleotide contents and relaxing effects on carbachol-induced contraction in the bovine abomasum. *J. Vet. Med. Sci.* **77**: 15–19. [Medline] [CrossRef]
7. Kaneda, T., Saito, E., Kanda, H., Urakawa, N. and Shimizu, K. 2015. Effects of papaverine on carbachol- and high K^+-induced contraction in the bovine abomasum. *J. Vet. Med. Sci.* **77**: 1269–1274. [Medline] [CrossRef]
8. Kaneda, T., Takeuchi, Y., Matsui, H., Shimizu, K., Urakawa, N. and Nakajyo, S. 2005. Inhibitory mechanism of papaverine on carbachol-induced contraction in bovine trachea. *J. Pharmacol. Sci.* **98**: 275–282. [Medline] [CrossRef]
9. Kaneda, T., Kubota, T., Fujimoto, K., Urakawa, N., Nakajyo, S. and Shimizu, K. 2010. Effects of rolipram on U46619-induced contraction and cyclic nucleotide content in the porcine coronary artery. *J. Smooth Muscle Res.* **46**: 17–29. [Medline] [CrossRef]
10. Kazić, T. 1977. Action of methylxanthines and imidazole on the contractility of the terminal ileum of the guinea pig. *Eur. J. Pharmacol.* **41**: 103–111. [Medline] [CrossRef]
11. Kenakin, T. P. 1984. The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.* **36**: 165–222. [Medline]
12. Keravis, T. and Lugnier, C. 2012. Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments. *Br. J. Pharmacol.* **165**: 1288–1305. [Medline] [CrossRef]
13. Moritoki, H., Morita, M. and Kanbe, T. 1976. Effects of methylxanthines and imidazole on the contractions of guinea-pig ileum induced by transmural stimulation. *Eur. J. Pharmacol.* **35**: 185–198. [Medline] [CrossRef]
14. Nassenstein, C., Wiegand, S., Lips, K. S., Li, G., Klein, J. and Kummer, W. 2015. Cholinergic activation of the murine trachealis muscle via non-vesicular acetylcholine release involving low-affinity choline transporters. *Int. Immunopharmacol.* **29**: 173–180. [Medline] [CrossRef]
15. Ozaki, H., Kwon, S. C., Tajimi, M. and Karaki, H. 1990. Changes in cytosolic CA^{2+} and contraction induced by various stimulants and relaxants in canine tracheal smooth muscle. *Pflugers Arch.* **416**: 351–359. [Medline] [CrossRef]
16. Panettieri, R. A. Jr., Kotlikoff, M. I., Gerthoffer, W. T., Hershenson, M. B., Woodruff, P. G., Hall, I. P., Banks-Schlegel S., National Heart, Lung, and Blood Institute 2008. Airway smooth muscle in bronchial tone, inflammation, and remodeling: basic knowledge to clinical relevance. *Am. J. Respir. Crit. Care Med.* **177**: 248–252. [Medline] [CrossRef]
17. Raeburn, D. and Advenier, C. 1995. Isoenzyme-selective cyclic nucleotide phosphodiesterase inhibitors: effects on airways smooth muscle. *Int. J. Biochem. Cell Biol.* **27**: 29–37. [Medline] [CrossRef]
18. Singh, M. and Sharma, P. L. 1985. Effect of cyclic nucleotide phosphodiesterase regulators on isolated rat uterus: with observations on their mechanism of action. *Arch. Int. Pharmacodyn. Ther.* **273**: 180–195. [Medline]
19. Zosky, G. R. and Sly, P. D. 2007. Animal models of asthma. *Clin. Exp. Allergy* **37**: 973–988. [Medline] [CrossRef]