J. Pharm. Pharmacol. 2000, 52: 1523–1528
Received January 20, 2000
Accepted June 22, 2000

Clonidine Attenuates the Carbachol-induced Contractile and Phosphatidylinositol Responses of Rat Trachea

OSAMU SHIBATA, MASATAKA SAITO, SHIGERU HASHIMOTO, KAZUYUKI SAKAI, TETSUJI MAKITA AND KOJI SUMIKAWA

Department of Anesthesiology, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan

Abstract

Although clonidine is known to affect vascular smooth muscle, its effects on airway smooth muscle are not fully understood. This study was designed to examine the effects of clonidine on carbachol-induced contractile and phosphatidylinositol responses of rat trachea.

Clonidine, at a dose of 100 μM or greater, attenuated carbachol-induced contraction and the accumulation of carbachol-induced inositol monophosphate (IP_1). Clonidine also attenuated the accumulation of aluminium fluoride-induced IP_1. The concentration–effect relationship of IP_1 accumulation was similar to that of carbachol-induced contraction; $r = 0{\cdot}797$, $P < 0{\cdot}001$.

These results suggest that clonidine attenuates contractile responses, at least in part, through the inhibition of phospholipase C (coupled with G-proteins) in phosphatidylinositol responses.

Clonidine affects the CNS, causing sedation and analgesia, and it also affects vascular smooth muscle. In the presence of electrical field stimulation, the contractile response of airway smooth muscle in the guinea-pig (Wikberg et al 1982), the dog (Tsuchiya et al 1990) and the horse (Yu et al 1993) were attenuated in a dose-dependent manner by clonidine. Lindgren et al (1986) reported that inhaled clonidine was beneficial to asthmatic patients. However, the mechanism involved in the clonidine-induced attenuation of tracheal smooth muscle contraction is not fully understood. Arimitsu et al (1998) reported that in carbachol-stimulated bovine tracheal smooth muscle, clonidine decreased intracellular Ca^{2+} and muscle tension in parallel. In addition, he reported that clonidine inhibited the transient increase in intracellular Ca^{2+} induced by carbachol in Ca^{2+}-free solution. Inositol 1,4,5-trisphosphate (IP_3) mobilizes Ca^{2+} from sarcoplasmic reticulum (Berridge 1983) and at the same time Ca^{2+} influxes from extracellular space. Thus, clonidine may inhibit phosphatidylinositol responses, resulting in the inhibition of Ca^{2+} influx and Ca^{2+} release, and subsequent airway smooth muscle relaxation. This study was designed to examine whether clonidine could attenuate the contractile and phosphatidylinositol responses of rat trachea.

Correspondence: O. Shibata, Department of Anesthesiology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
E-Mail: opshiba@net.nagasaki-u.ac.jp

Materials and Methods

Drugs

Carbachol, clonidine, glibenclamide and aluminium fluoride were purchased from Sigma (St Louis, MO) and [^{3}H]*myo*-inositol was purchased from Amersham (Tokyo, Japan).

Animals

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

Contractile response

Each trachea was cut into 3-mm-wide ring segments with a McIlwain tissue chopper (The Mickle

Laboratory Engineering, Gomshall, UK). Each tracheal ring segment was suspended between two stainless hooks and placed in a 5-mL water-jacketed organ chamber (Kishimotoika, Kyoto Japan) containing Krebs-Henseleit solution (composition in mM: 118 NaCl, 4·7 KCl, 1·3 $CaCl_2$, 1·2 KH_2PO_4, 1·2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose, 0·05 Na_2-EDTA). The solution was continuously aerated with O_2 95%/CO_2 5% at a temperature of 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). The resting tension was adjusted periodically to 1·5 g during the equilibration period. The ring was washed every 15 min and re-equilibrated to baseline tension for 60 min (time 0).

We determined the carbachol concentration at 80% of the level of maximal contraction of the rat tracheal rings. At time 0, ring contraction was induced by stepwise cumulative additions of carbachol (0–100 μM in final concentrations). The effects of clonidine on the carbachol-induced contraction of rat tracheal ring in the presence or absence of glibenclamide (a potassium-channel blocker) or aluminium fluoride (G-protein stimulator) (Sternweis & Gilman 1982; Chabre 1989) were then examined. At time 0, carbachol (0·55 μM in final concentration) was added and 30 min later, glibenclamide (10μM in final concentration) or aluminium fluoride (100 μM in final concentration) was added. After a period of 15 min, ring relaxation was induced by stepwise cumulative additions of clonidine (0–300 μM in final concentrations).

Phosphatidylinositol response

The technique of Brown et al (1984) was used. Inositol 1,4,5-trisphosphate (IP_3) is rapidly degraded into IP_1 and this is recycled back to phosphatidylinositol via free inositol (Figure 1). Lithium inhibits the conversion of IP_1 to inositol. Thus, in the presence of Li^+, the accumulation rate of IP_1 reflects the extent of phosphatidylinositol response. We measured [^{3}H]IP_1 in tracheal slices incubated with [^{3}H]*myo*-inositol. The trachea was cut longitudinally and chopped into 1-mm-wide pieces with a McIlwain tissue chopper. Three pieces of the tracheal slices were placed in small flat-bottomed tubes and pre-incubated for 15 min in Krebs-Henseleit solution containing 5 mM LiCl. The solution was continuously aerated with 95% O_2/5% CO_2. A sample of 0·5 μCi [^{3}H]*myo*-inositol was then added to each tube (final concentration 0·1 μM in a 300-μL incubation volume). All of the tubes were subsequently flushed with 95% O_2/5% CO_2, capped, set in a shaking bath at 37°C and incubated for 30 min (time 0).

We also examined the effects of clonidine on carbachol-induced IP_1 accumulation in rat tracheal slices. At time 0, varying doses (0, 30, 100 and 300 μM) of clonidine were added to the tracheal-slice suspensions and the tubes were flushed with 95% O_2/5% CO_2. After a period of 15 min, carbachol (0·55 μM in final concentration) was added. After an additional 60 min, the reaction was stopped with 940 μL 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* over a period of 5 min.

It has been suggested that aluminium fluoride stimulates G-proteins (Sternweis & Gilman 1982; Chabre 1989) produces IP_3 (Wood et al 1989). It is not clear whether clonidine affects G-protein-coupled phospholipase C. We examined the effects of clonidine on aluminium fluoride-induced IP_1 accumulation, and carbachol-induced IP_1 accumulation in the presence of aluminium fluoride. At time 0, clonidine (300 μM in final concentration) was added to the suspension of tracheal slices and the tubes were flushed with 95% O_2/5% CO_2, and 15 min later, carbachol (0·55 μM in final concentration) and aluminium fluoride (100 μM in final concentration) was added. After an additional 60 min, the reaction was stopped with 940 μL chloroform : methanol (1 : 2 v/v) as described above.

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 the formate form. The [^{3}H]IP_1 formed in the tracheal slices was counted with a liquid scintillation counter and the counts were measured in becquerels (Bqs). The Bq counts of the blank samples (no slices present) were subtracted from all of the other counts to obtain the experimental data.

Statistical analysis

Data were expressed as mean ± s.e.m. The results of repeated measures and multiple groups were analysed using two-way variance analysis. Multiple pairwise comparisons between groups were assessed by Scheffe's test. $P < 0.05$ was considered significant.

Results

Figure 2 shows a typical recording of the effects of clonidine on carbachol-induced contraction of a rat

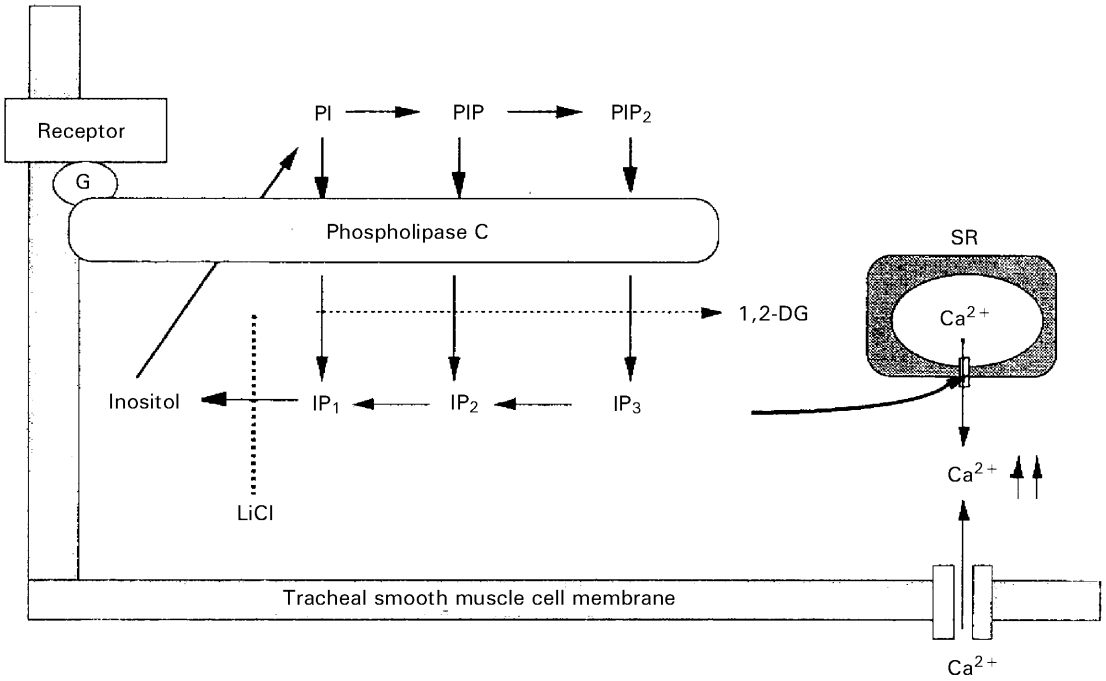

Figure 1. Schematic diagram of the phosphatidylinositol pathway. PI, phosphatidylinositol; P, phosphate; DG, diacyl glycerol; SR, sarcoplasmic reticulum; G, G protein; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol 1,4,5-triphosphate.

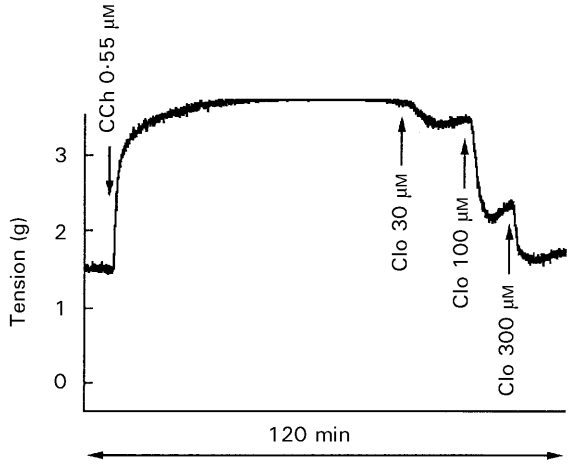

Figure 2. A typical recording of the effects of clonidine on carbachol-induced contraction of rat trachea. CCh, carbachol; Clo, clonidine.

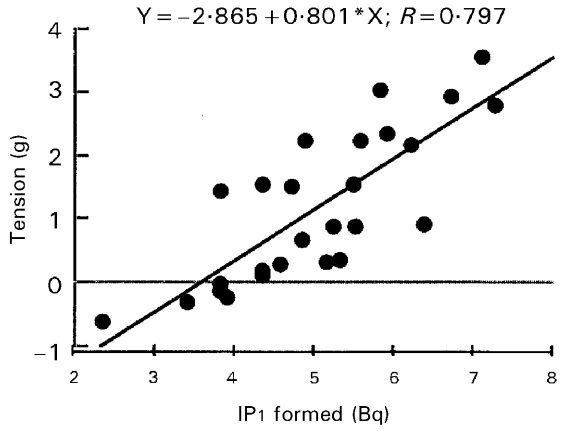

Figure 3. Relationship between carbachol-induced IP_1 accumulation and carbachol-induced contraction. Bq, becquerel.

tracheal ring. Table 1 shows that clonidine attenuated carbachol-induced tracheal-ring contraction at a dose of 100 μM or greater ($P < 0{\cdot}01$). Neither glibenclamide nor aluminium fluoride affected carbachol-induced contraction. In addition, neither compound affected the clonidine's inhibition of carbachol-induced contraction. Table 2 indicates that clonidine attenuated 0·55 μM carbachol-induced IP_1 accumulation at a dose of 300 μM ($P < 0{\cdot}05$). Table 3 indicates that aluminium fluoride was observed to stimulate IP_1 accumulation, and that clonidine attenuated aluminium fluoride-induced IP_1 accumulation in the presence or absence of carbachol. The concentration–effect relationship of IP_1 accumulation was similar to that of carbachol-induced contraction; $r = 0{\cdot}797$, $P < 0{\cdot}001$ (Figure 3).

Discussion

In this study, we found that clonidine attenuates the carbachol-induced contractile and PI responses of rat trachea, even in the presence of aluminium

Table 1. Effects of clonidine on carbachol-induced tension of rat trachea in the presence of glibenclamide or aluminium fluoride.

Tension (g)	Clonidine (μM)				
	0	10	30	100	300
Control (carbachol 0·55 μM)	1·92 ± 0·23	1·84 ± 0·23	1·57 ± 0·26	0·46 ± 0·26*	0·02 ± 0·19**
Glibenclamide 10 μM	1·87 ± 0·12	1·71 ± 0·21	1·37 ± 0·29	0·53 ± 0·29*	−0·05 ± 0·12**
Aluminium fluoride 100 μM	1·77 ± 0·16	1·62 ± 0·18	1·12 ± 0·25	0·32 ± 0·20**	−0·09 ± 0·09**

Values are expressed as mean ± s.e.m., n = 6–7. *$P < 0·01$, **$P < 0·001$, compared with clonidine 0 μM.

Table 2. Effects of clonidine on carbachol-induced IP_1 accumulation of rat trachea.

	Clonidine (μM)			
	0	30	100	300
IP_1 accumulation (Bq)	6·25 ± 0·54	5·51 ± 0·51	4·80 ± 0·39	4·22 ± 0·38*

Values are expressed as mean ± s.e.m., n = 6–8. *$P < 0·05$ compared with clonidine 0 μM.

Table 3. Effects of clonidine on aluminium-fluoride-induced IP_1 accumulation in the presence or absence of carbachol.

	Basal	Aluminium fluoride 100 μM			
		Alone	+ Clonidine 300 μM	+ Carbachol 0·55 μM	+ Clonidine + carbachol
IP_1 accumulation (Bq)	2·07 ± 0·24	4·26 ± 0·27**	2·24 ± 0·13††	6·88 ± 0·38**	4·59 ± 0·35##

Values are expressed as mean ± s.e.m., n = 6–8. **$P < 0·001$ vs basal, ††$P < 0·001$ vs aluminium fluoride, ##$P < 0·001$ vs aluminium fluoride + carbachol.

fluoride. In addition, we found that clonidine's effect on carbachol-induced phosphatidylinositol responses is consistent with that on carbachol-induced contraction. The action of clonidine on airway smooth muscle may therefore involve one of the following mechanisms. Firstly, clonidine may inhibit carbachol-induced acetylcholine release from postganglionic nerve terminals, resulting in attenuation of tracheal smooth muscle contraction. Since parasympathetic postganglionic neurons are considered to be close to targeted end-organs, the tracheal rings used in this study would contain parasympathetic postganglionic neurons. Although carbachol has muscarinic action, it retains substantial nicotinic activity, particularly on autonomic ganglia. Thus, contractile responses to carbachol may be due, in part, to the activation of nicotinic receptors on parasympathetic post-ganglionic neurons. Yu et al (1993) observed that clonidine dose-dependently inhibited acetylcholine release and the contractile response to electrical field stimulation. This inhibition was attenuated by the α_2-adrenoceptor antagonists yohimbine and idazoxan, but not by the α_1-adrenoceptor antagonist prazosin. Yu et al (1993) concluded that α_2-adrenoceptors exist on cholinergic nerves innervating airway smooth muscle, and activation of these receptors by clonidine would inhibit cholinergic neurotransmission. However, 1,1-dimethyl-4-phenyl-piperazinium, a selective ganglionic nicotinic agonist, did not cause contraction of rat tracheal rings (Shibata et al 1998). The preparation does not appear to contain a sufficient number of functional postganglionic cells which can be activated by nicotinic agonists. Thus, clonidine's inhibition of the carbachol-induced contraction of rat trachea could not be due to the inhibition of acetylcholine release.

A second possible mechanism for the action of clonidine on airway smooth muscle could be via activation of ATP-sensitive K^+ channels, resulting in attenuation of tracheal smooth muscle contraction. Ishiyama et al (1998) reported that glibenclamide, an ATP-sensitive K^+-channel blocker, potentiated clonidine-induced arterial contraction. Silva et al (1996) reported that clonidine caused hyperpolarization which was inhibited by yohimbine or idazoxan, but not by prazosin. This

hyperpolarization was also abolished by glibenclamide. Silva et al (1996) concluded that clonidine could open K^+ channels and hyperpolarize the plasma membrane of the mesenteric artery by acting on α_2-adrenoceptors. However, in this study, glibenclamide could not abolish the attenuation caused by clonidine on carbachol-induced tracheal smooth muscle contraction. Thus, clonidine could not cause activation of ATP-sensitive K^+ channels of rat tracheal smooth muscle.

A third possible mechanism of action is that clonidine may inhibit the carbachol-induced phosphatidylinositol response, resulting in attenuation of tracheal smooth muscle contraction. When muscarinic receptors in the airway smooth muscle cell membranes are stimulated by carbachol to activate phospholipase C, phosphatidylinositol-4,5-bisphosphate (PIP_2) is hydrolysed into IP_3 and diacylglycerol. IP_3 mobilizes Ca^{2+} from sarcoplasmic reticulum and, at the same time, an influx of Ca^{2+} occurs from the extracellular space (Berridge 1983). Diacylglycerol activates protein kinase C which may also be a mechanism of modulating or controlling airway smooth muscle tension. Clonidine was reported to inhibit a transient increase in intracellular Ca^{2+} induced by carbachol, but not by caffeine in Ca^{2+}-free solution. In addition, clonidine decreased intracellular Ca^{2+} and muscle force in parallel in carbachol-induced bovine tracheal smooth muscle (Arimitsu et al 1998). In our present study, clonidine attenuated carbachol-induced IP_1 accumulation and the effect on carbachol-induced IP_1 accumulation was consistent with that on carbachol-induced contraction. However, clonidine could not completely inhibit the carbachol-induced phosphatidylinositol response, even at 300 μM. However, it could inhibit carbachol-induced tracheal smooth muscle contraction at the same dose. Arimitsu et al (1998) reported that clonidine diminished the transient intracellular Ca^{2+} rise elicited by carbachol by approximately 50%. Thus, a phosphatidylinositol response would be involved in an attenuation by clonidine of carbachol-induced contraction of rat tracheal rings.

It has been reported that the phospholipase C coupled to G-proteins is stimulated by aluminium fluoride and that aluminium fluoride induces IP_3 formation (Sternweis & Gilman 1982; Chabre 1989; Wood et al 1989). In this study, aluminium fluoride stimulated a phosphatidylinositol response and this response was attenuated by clonidine. Thus, clonidine would inhibit G-protein-coupled phospholipase C in phosphatidylinositol responses, resulting in an attenuation of contractile responses of rat trachea.

A fourth possibility is that clonidine may also act on imidazoline receptors, resulting in an attenuation of tracheal smooth muscle contraction, because the effect of clonidine on the carbachol-induced phosphatidylinositol response was only partial. Arimitsu et al (1998) reported that the relaxant effect of clonidine was inhibited by idazoxan and yohimbine. This suggests the involvement of α_2-adrenoceptors in the relaxant effect of clonidine. However, imidazoline receptors are reported to exist in smooth muscle cells (Yablonsky & Dausse 1991; Regunathan et al 1995). Arimitsu et al (1998) also reported that phentolamine, an imidazoline receptor agonist, caused dose-dependent relaxation of carbachol-induced contraction, and that their effects were inhibited by idazoxan but not by yohimbine. Idazoxan has a much higher affinity for imidazoline receptors than does yohimbine (Szabo & Urban 1995). Thus, it seems probable that clonidine would, in part, act on imidazoline receptors, resulting in an attenuation of tracheal smooth muscle contraction.

In conclusion, clonidine inhibited carbachol-induced contractile responses and it attenuated carbachol-induced IP_1 accumulation even in the presence of aluminium fluoride. These results suggest that clonidine inhibits contractile responses, in part, through the inhibition of G-protein-coupled phospholipase C in phosphatidylinositol responses.

Acknowledgements

This study was supported in part by Grants-in Aid for Scientific Research C, No. 10671421 from the Ministry of Education, Science and Culture, Japan.

References

Arimitsu, M., Mitsui, S. M., Sato, K. (1998) Mechanism of relaxant effect of clonidine in isolated bovine tracheal smooth muscle. J. Pharmacol. Exp. Ther. 286: 681–687

Berridge, M. J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyze polyphosphoinositides instead of phosphatidylinositol. Biochem. J. 212: 849–858

Brown, E., Kendall, D. A., Nahorski, S. R. (1984) Inositol phospholipid hydrolysis in rat cerebral cortical slices: I. Receptor characterization. J. Neurochem. 42: 1379–1387

Chabre, M. (1989) Aluminofluoride action on G-proteins of the adenylate cyclase system is not different from that on transducin [letter]. Biochem. J. 258: 931–932

Ishiyama, T., Dohi, S., Iida, H. (1998) The vascular effects of topical and intravenous alpha 2-adrenoceptor agonist clonidine on canine pial microcirculation. Anesth. Analg. 86: 766–772

Lindgren, B., Ekstrom, T., Anderson, R. (1986) The effect of inhaled clonidine in patients with asthma. Am. Rev. Respir. Dis. 134: 266–269

Regunathan, S., Youngson, C., Wang, H. (1995) Imidazoline receptors in vascular smooth muscle and endothelial cells. Ann. N.Y. Acad. Sci. 763: 580–590

Shibata, O., Tsuda, A., Makita, T. (1998) Contractile and phosphatidylinositol responses of rat trachea to anticholinesterase drugs. Can. J. Anaesth. 45: 1190–1195

Silva, E. G., Feres, T., Vianna, L. M. (1996) Dual effect of clonidine on mesenteric artery adrenoceptors: agonistic (alpha 2) and antagonistic (alpha 1). J. Pharmacol. Exp. Ther. 277: 872–876

Sternweis, P. C., Gilman, A. G. (1982) Aluminium: a requirement for activation of the regulatory component of adenylate cyclase by fluoride. Proc. Natl Acad. Sci. USA 79: 4888–4891

Szabo, B., Urban, R. (1995) Mechanism of sympathoinhibition by imidazolines. Ann. N.Y. Acad. Sci. 763: 550–565

Tsuchiya, Y., Hosokawa, T., Kasuya, Y. (1990) Involvement of alpha 2-adrenergic receptors in the vagal reflex-induced tracheal constriction. J. Pharmacobiodyn. 13: 30–35

Wikberg, J. E., Grundstrom, N., Visnovsky, P. (1982) Pharmacology of B-HT 920 in some isolated smooth muscles of the guinea-pig. Acta Pharmacol. Toxicol. 50: 266–271

Wood, S. F., Szuts, E. Z., Fein, A. (1989) Inositol trisphosphate production in squid photoreceptors. Activation by light, aluminum fluoride, and guanine nucleotides. J. Biol. Chem. 264: 12970–12976

Yablonsky, F., Dausse, J. P. (1991) Non-adrenergic binding sites for the "alpha 2-antagonist" [^{3}H]idazoxan in the rabbit urethral smooth muscle. Pharmacological and biochemical characterization. Biochem. Pharmacol. 41: 701–707

Yu, M., Wang, Z., Robinson, N. E. (1993) Prejunctional alpha 2-adrenoceptors inhibit acetylcholine release from cholinergic nerves in equine airways. Am. J. Physiol. 265: L565–L570