Review Article

In vitro reactivity ("organ chamber") of guinea pig tracheal rings—methodology considerations

Agnes Afrodite Sumarelli Albuquerque[1], Marco Túlio Menezes Carvalho[1], Patricia Martinez Evora[2], Tales Rubens de Nadai[1], Andrea Carla Celotto[1], Paulo Roberto Barbosa Evora[1]

[1]Department of Surgery and Anatomy, Ribeirão Preto School of Medicine, University of São Paulo, Avenida Bandeirantes 3900, 14048-900 Ribeirão Preto, SP, Brazil; [2]Department of Animal Pathology, Veterinary School, São Paulo State University-UNESP, Campus of Jaboticabal, Brazil

Contributions: (I) Conception and design: PR Evora, AC Celotto; AA Albuquerque; (II) Administrative support: AA Albuquerque; (III) Provision of study materials or patients: AA Albuquerque; (IV) Collection and assembly of data: AA Albuquerque; (V) Data analysis and interpretation: PR Evora, TR de Nadai, MT Carvalho; (VI) Manuscript writing: PM Evora; (VII) Final approval of manuscript: All authors.

Correspondence to: Paulo Roberto Barbosa Evora. Rua Rui Barbosa, 367. Apt. 15, 14015-120 Ribeirão Preto, São Paulo, Brazil. Email: prbevora@gmail.com.

Abstract: The present text was motivated by the difficulties faced by our postgraduate students when using airways studies protocols and will take into consideration the three mechanisms of relaxation: (I) guanosine 3,5-cyclic monophosphate (cGMP)/NO-dependent; (II) adenosine 3,5-cyclic monophosphate (cAMP)/PGI2-dependent, and (III) hyperpolarization-dependent. Tracheal rings are studied in an organ bath containing a gassed physiological salt solution, usually at a temperature of 37 ℃. An agent or procedure that causes contraction [acetylcholine (Ach) or metacholine] of the smooth muscle is needed before study airway dilator drugs. The presented airways studies protocols are useful to study the bronchial epithelial-dependent reactivity.

Keywords: Trachea; tracheal *in vitro* reactivity; organ-chambers; airway epithelium; guinea pig

Submitted Mar 16, 2016. Accepted for publication Apr 06, 2016.
doi: 10.21037/atm.2016.05.18
View this article at: http://dx.doi.org/10.21037/atm.2016.05.18

Introduction

This review was designed and conducted to, briefly and precisely, describe the "organ chambers" techniques for *in vitro* experimentation on isolated trachea preparations. The "organ chambers" experiments and protocols are usually complex and diverse, and technical publications are scarce. One of the best, if not the best, publication about *in vitro* methods to study pharmacological airway properties is authored by Fedan and colleagues [2001] (1). Thus, this study became the "master" text of this review, combined with the Hall paper about the airway pharmacology (2), Moreover, only the "organ chambers" preparations that we used in our laboratory will be highlighted.

The present text was motivated by the difficulties faced by our postgraduate students when using airways studies protocols and will take into consideration the three mechanisms of relaxation: (I) guanosine 3,5-cyclic monophosphate (cGMP)/NO-dependent; (II) adenosine 3, 5-cyclic monophosphate (cAMP)/PGI2-dependent; and (III) hyperpolarization-dependent.

The pharmacological basis

Mechanisms of airway smooth muscle (ASM) relaxation

The cGMP/NO pathway

The cGMP and cyclic cAMP are second messengers capable of mediating the actions of a variety of drugs and hormones. There is a large body of evidence supporting the hypothesis that cAMP is the main airways mediator of the ASM drugs relaxant effects, such as alpha 2-adrenoceptor agonists (3). Although most attention has been focused on cAMP signal transduction pathways, recent evidence suggests that cGMP pathway is also an important second messenger with important relaxant and antiproliferative

Table 1 Mechanisms underlying ASM relaxation by β2-agonists [Reprinted with permission (2)]

cAMP-dependent
Inhibition of inositol phospholipid hydrolysis
Stimulation of calcium activated potassium channels (K_{Ca})
Membrane hyperpolarization
Inhibition of myosin light chain kinase activation
Alteration of phosphorylation state of contractile apparatus
Increased Ca^{2+} reuptake/extrusion
cAMP-independent
Stimulation of K_{Ca} by α subunit o stimulatory G protein (Gsα)

ASM, airway smooth muscle; cAMP, adenosine 3,5-cyclic monophosphate.

effects (3). *In vitro* and *in vivo* studies shows, in agreement with the cGMP role, that nitric oxide (NO) relaxes ASM with intermediate potency between the alpha-adrenoceptor agonist isoprenaline and the phosphodiesterase (PDE) inhibitor theophylline. In addition, epithelium-derived relaxing factor (EpDRF), an NO or NO-like substance, is an ASM relaxant in some species.

As already mentioned, agents that elevate cGMP levels can relax ASM preparations in organ bath systems. However, the potential importance of cGMP in ASM *in vivo* relaxant responses (as opposed to vascular) is unclear.

The cAMP pathway

The ASM cell is the primary effector cell governing the control of airway caliber in the human lung. The contractile state of the ASM cell is predominantly influenced by the balance of constrictor and relaxant stimuli. Agents such as histamine and acetylcholine (Ach) cause contraction, activating specific cell surface receptors engaged with signal transduction pathways and ion channels. The predominant contraction pathway is the phospholipase C (PLC) activation with increasing of intracellular calcium levels. Relaxation is mediated predominantly by stimulation of adenylyl cyclase-coupled receptors (e.g., the β-adrenoceptor) resulting in elevation of cell cAMP content (*Table 1*). Complex crosstalk occurs between these pathways and also ion channels expressed on the cell membrane, leading to careful regulation of ASM muscle tone (2).

Hyperpolarization and ASM reactivity

Various types of potassium (K^+) channels are expressed by ASM cells, which play a crucial role in determining the resting membrane potential, electrical stability and responsiveness to both contractile and relaxant agents. Moreover, K^+ channels are also involved in modulation of neurotransmitter release from airway nerves. The most important K^+ channels identified in airways include large and small Ca^{2+}-activated, delayed-rectifier, and ATP-sensitive channels. These K^+ channels are structurally and functionally different, thus playing distinct roles in airway electrophysiology and pharmacology. Many *in vitro* and *in vivo* studies, performed in both animals and humans, have shown that K^+ channel openers can induce hyperpolarization of ASM cells, bronchodilation, suppression of airway hyperresponsiveness (AHR), and inhibition of neural reflexes.

Most of the studies conducted to evaluate the effects of K1 channel opening on ASM contractility have been performed using KATP channel activators; at present, only a few molecules are known to selectively open maxi-K_{Ca} channels. *In vitro* studies have shown that KATP channel activators have some spasmolytic activity on ASM of several different species. In contrast, these drugs do not exert a significant protective effect against the response induced by high concentrations of contractile agonists. Moreover, there are significant differences among species concerning the protective action of KATP channel openers against bronchoconstrictive stimuli. In guinea pig ASM, these compounds show a reduced inhibitory effect on contractile responses elicited by cholinergic agents. In comparison with β-adrenoceptor agonists, KATP channel activators have a less potent *in vitro* relaxing activity on ASM, suggesting a questionable relevance (4).

Mechanisms of ASM contraction

Pathways involved in ASM contraction are quite similar to those participating in vascular vessels. Following binding of agonist to receptor in the cell membrane, the associated G protein which exists as a heterotrimeric complex of a, b and c subunit dissociates, and the free a subunit stimulates PLC, which in turn catalyses the breakdown of phosphatidylinositol 4,5 biphosphate (PIP2), resulting in the formation of the two intracellular messengers diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). IP3 can release

Table 2 Contractile agonists in ASM and their receptors [Reprinted with permission (2)]

Agonist (Receptor)
Histamine (H1)
Ach (M3; M2 also present, role unclear)
Leukotriene D4 (LTD4)
Substance P (? NK2)
Bradykinin (B2; atypical B3 receptors ?)
5-Hydroxytryptamine (? 5-HT2 Subtype not fully defined)

?, means "not fully defined". ASM, airway smooth muscle; Ach, acetylcholine.

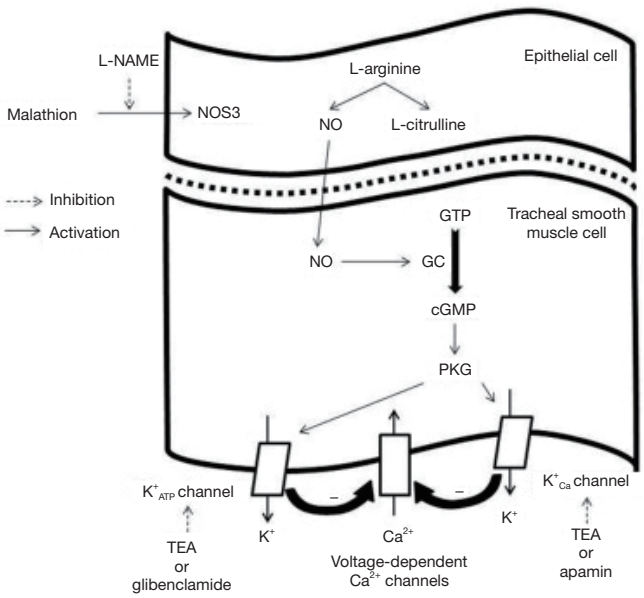

Figure 1 Agonist activates nitric oxide synthase 3 (NOS3) isoform that up-regulates the production of nitric oxide (NO) in the epithelial cell. NO activates guanylate cyclase (GC), which increases the level of intracellular cyclic GMP. Increased cGMP then activates the protein kinase G (PKG) cascade, enhancing K^+ efflux and attenuating Ca^{2+} influx-associated smooth muscle cell contractility (5). GTP, guanosine triphosphate; L-NAME, N-nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one; TEA, tetraethylammonium; cGMP, guanosine 3,5-cyclic monophosphate.

calcium from intracellular stores via the IP3 receptor (IP3R), whereas DAG can stimulate calcium entry in addition to activating protein kinase C. The rise in cytosolic free calcium levels leads to contraction of the ASM. Some other contractile agonists coupled to receptors expressed on ASM are also able to stimulate contractile responses through the same intracellular signaling pathway. These agonists and the receptors through which they operate are shown in *Table 2* and *Figure 1* (2).

Role of epithelium

The airway epithelium removal affects the contractile and relaxant smooth muscle reactivity under *in vitro* experiments. Differently from the vascular endothelium, a non-NO and nonprostanoid factor named EpDRF, the airway reactivity is not explained by the epithelium barrier remotion (1). A variety of methods have been used to remove the epithelium, and they have been adapted to the size of the airway being studied. In large airways, for example, rubbing the surface with a cotton-tipped applicator is effective, but other gentle abrasives are equally valid. It is important that the epithelium is removed without damaging the smooth muscle.

The "organ chamber" setup

Muscle strips or tracheal rings are studied in an organ bath ("organ-chamber") containing a gassed physiological salt solution. We prefer to consider tracheal rings because of their easier instrumental feasibility. Several solutions are used, which differ in the composition and concentrations of the salts and buffer systems employed. A bicarbonate-containing solution requires CO_2 gassing to set the pH. The solution widely used is a modified Krebs-Henseleit solution, which consists of (mM): NaCl (113.0), KCl (4.8), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25.0), and glucose (5.7), pH 7.4 (37 ℃); gassed with 95% O_2 + 5% CO_2. The temperature is usually kept at 37 ℃, but this may vary depending on the body temperature of the animal from which the tissue was derived, or other experimental needs. Allowing an ample equilibration period at 37 ℃ allows the muscle to generate ATP, reactivate ATP-dependent transport and restore resting

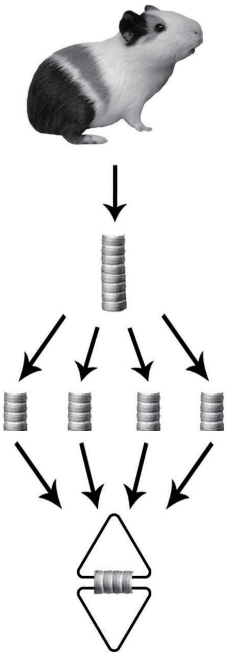

Figure 2 The animals are housed under standard laboratory conditions (12 h light/dark cycle at 21 ℃), with free access to food and water. Under anesthesia, laparotomy is carried out for exsanguination via the abdominal aorta and a thoraco-cervicotomy is done for the tracheal harvesting. The trachea is carefully dissected free of connective tissue and immediately immersed in Krebs solution (Composition (mM) is: NaCl –118.0, KCl –4.7, $CaCl_2$ –2.5, KH2PO4 –1.2, $MgSO_4$ –1.66, glucose –11.1, $NaHCO_3$ –25.0 (pH 7.4). The trachea is cut into rings (4–5 mm in length). The epithelium is removed from some rings by gently rubbing the intimal surface of the blood vessel with a pair of watchmaker's forceps.

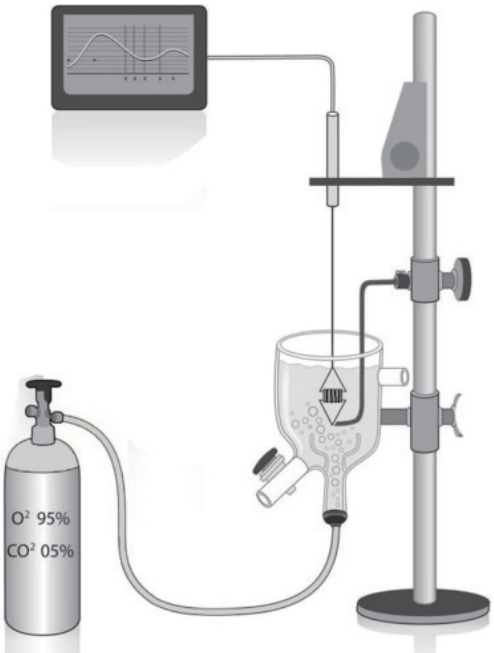

Figure 3 Schematic illustration of the "organ chamber" setup. Tracheal rings were placed in isolated organ baths (10 mL) filled with Krebs solution, maintained at 37 ℃ and bubbled with 95% O_2/5% CO_2 (pH 7.4). Each arterial ring was suspended by two stainless steel clips placed through the lumen. One clip was anchored to the bottom of the organ bath while the other was connected to a strain gauge for measurement of the isometric. Each ring was stretched to a resting tension of 1.5 g and allowed to equilibrate for 60 min. During this period, tissues were washed every 15 min.

membrane potential. This detail is mandatory if the tissue is stored in an iced solution or remains on laboratory room ambient for a long period.

- To measure responses of the smooth muscle preparation, one ligature or wire assembly is attached to a fixed holder at the bottom of the organ bath containing Krebs solution, while the other is attached to a force transducer to measure mechanical responses (i.e., contraction or relaxation) of the smooth muscle (*Figures 2,3*);
- After set up, the preparation is elongated to place it at a length (Lo) in which it develops maximal contractile force. While monitoring the output of the transducer on the chart recorder or computer screen, the preparations are slowly lengthened to remove slack in the connections between the anchor and the transducer and to reach a predetermined optimum passive or baseline force (i.e., tension);
- Following the application of passive force, a mandatory incubation period of at least 1 h should follow before any experimental intervention begins. The solution should be changed, at regular intervals (15 min), to prevent the buildup of metabolic products. This incubation period is also necessary to allow the muscle to reestablish the normal ion content that goes awry during tissue preparation. If many investigators use a 1 g optimum passive force on a like preparation to establish a baseline, it will not make sense to begin a length–tension study by applying 2 g of force at the outset. It is always important to emphasize that over adjusting the amount of applied force may eventually damage the

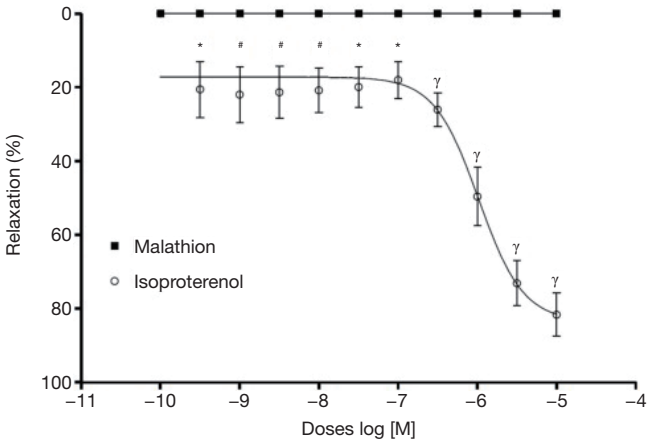

Figure 4 Curve concentration-effect to malathion and isoproterenol (10_{-10} to 10_{-6} M) in guinea pig trachea. * indicates significant difference (P<0.05) between the malathion group compared to isoproterenol group. # indicates significant difference (P<0.01) between the malathion group compared to isoproterenol group. ɣ indicates significant difference (P<0.001) between the malathion group compared to the isoproterenol group. Values represent the mean ± standard deviation (n=7). Two-way ANOVA, post-test Bonferroni.

smooth muscle;

- An agent or procedure that causes contraction of the muscle is needed. The choice can be eased by the pharmacological properties of the preparation as reported in the literature. Histamine or Ach (or analogs of Ach such as methacholine or carbachol) are useful agonists for this purpose (histamine does not contract rat airways). A concentration must be chosen for use and we recommend that it is less than or equal to the EC50 value for the agonist; use of high concentrations of these agents (10^{-4} M) can result in desensitization of the muscle and spurious results.

Note: the presence of a spontaneous tone in the preparation may complicate the establishment of basal conditions. The spontaneous tone reflects an intrinsic activation of the smooth muscle, which the investigator has not caused and, therefore, is probably due to the effects of released prostanoids (1).

The "organ chamber" setup is illustrated in *Figures 2-4*. Our protocol is summarized in *Table 3*.

Table 3 Study protocol of the trachea reactivity in organ chambers

Removal of the trachea of anesthetized animals with an intraperitoneal injection of sodium thiopental (40 mg/kg body weight)
After removal carefully to avoid damaging the thread, it is immediately immersed in Krebs solution
Section of the tracheal rings of 4–5 mm (two cartilaginous rings proximal to the lungs)
After this initial preparation, each ring is suspended between two stainless steel clips, passed through its lumen, and this assembly is immersed in a vat system for tissue isolated organ bath
The wells are filled with 10 mL of Krebs solution (the same composition used to the aorta) maintained at 37 ℃ and bubbled with a mixture cariogenic (95% O_2 and 5% CO_2). One of the handles is anchored to a fixed support, and the other connected to a transducer (Grass force-displacement transducer FT03, Grass Instrument CO, Quincy, USA) for measurement of isometric force. The transducers are then coupled to an 8-channel recorder (Gould, Cleveland, USA), thus allowing the simultaneous recording of isometric tension
The tracheal rings are subjected to optimum length-tension of 1.5 g, using a micrometer system, and remain in this resting tension for 60 minutes, before starting the experiments with pharmacological agents.
After this period of stabilization, the contraction of the rings is induced by the addition of ACh (10^{-4} M) to the bath
The rings were relaxed with isoproterenol (10^{-5} M) after reaching the plateau of contraction and are only used those with at least 50% relaxation
Subsequently, the solution vat was replaced with a Krebs solution, and pure preparation was allowed to stand for 20 minutes
After this second period of stabilization, concentration-response curves in cumulative concentrations of the substance to be tested (10^{-10} to 10^{-6} M) and Isoproterenol (10^{-10} to 10^{-6} M), precontracted with ACh (10^{-4} M) and curves to Ach (10^{-10} to 10^{-6} M) preincubated with or without blockers. Note: the time of incubation for each minim inhibitor is 45 minutes

Ach, acetylcholine.

Acknowledgements

Sao Paulo Research Foundation (FAPESP), National Council of Scientific and Technological Development (CNPq), Coordination of Improvement of Higher Academic Staff (CAPES) Research performed at Laboratory of Cardiovascular and Endothelium Function, Ribeirao Preto School of Medicine, University of Sao Paulo (FMRP-USP), Ribeirao Preto-SP, Brazil. Part of PhD degree thesis, Experimental Medicine Program. Tutor: Paulo Roberto Barbosa Evora.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Fedan JS, Van Scott MR, Johnston RA. Pharmacological techniques for the in vitro study of airways. J Pharmacol Toxicol Methods. 2001;45:159-74.
2. Hall IP. Second messengers, ion channels and pharmacology of airway smooth muscle. Eur Respir J 2000;15:1120-7.
3. Hamad AM, Clayton A, Islam B, et al. Guanylyl cyclases, nitric oxide, natriuretic peptides, and airway smooth muscle function. Am J Physiol Lung Cell Mol Physiol 2003;285:L973-83.
4. Pelaia G, Gallelli L, Vatrella A, et al. Potential role of potassium channel openers in the treatment of asthma and chronic obstructive pulmonary disease. Life Sci 2002;70:977-90.
5. Vieira AB, Coelho LP, Insuela DB, et al. Mangiferin prevents guinea pig tracheal contraction via activation of the nitric oxide-cyclic GMP pathway. PLoS One 2013;8:e71759.

Cite this article as: Albuquerque AA, Carvalho MT, Evora PM, de Nadai TR, Celotto AC, Evora PR. *In vitro* reactivity ("organ chamber") of guinea pig tracheal rings—methodology considerations. Ann Transl Med 2016;4(11):216. doi: 10.21037/atm.2016.05.18

