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Relaxation of tracheal smooth muscle independent on functional epithelium cells induced by lidocaine, bupivacaine and isomers in rats☆

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ABSTRACT

Lidocaine is a local anesthetic which has been used to protect spasm reaction during tracheal intubation and bronchoscopy. We compared the potency of lidocaine, bupivacaine (RS(±)-bupivacaine) and isomers (S(−)-bupivacaine and R(+)-bupivacaine) to promote relaxation of tracheal smooth muscle. Relaxation of airways smooth muscle can be dependent on the release of relaxing factors by epithelium such as prostanoids and nitric oxide (NO). Possible mechanisms involved in the tracheal smooth muscle relaxation induced by these local anesthetics were evaluated in preparation in which the epithelium layer was intact or denuded. Bupivacaine and its isomers were approximately six to eleven-fold more potent than lidocaine to promote relaxation on acetylcholine-induced contraction in tracheal rings. The concentration of lidocaine, RS(±)-bupivacaine, S(−)-bupivacaine and R(+)-bupivacaine necessary to produce a 50% reduction of maximal contraction to acetylcholine (IC_{50}) in tracheal rings with intact epithelium was 1.25 ± 0.01, 0.11 ± 0.01, 0.15 ± 0.01, 0.19 ± 0.01 mM, respectively. Removal of epithelium or exposure to N^G-nitro-L-arginine methyl ester, indomethacin did not alter the IC_{50}. However, calcium influx of depolarized tracheal smooth muscle was inhibited by lidocaine, bupivacaine and isomers. S(−)-bupivacaine reduced by 78.8 ± 7.4% the calcium influx followed by RS(±)-bupivacaine (41.8 ± 6.7%) and R(+)-bupivacaine (25.6 ± 9.5%). In conclusion, local anesthetic action was stereoselective and partially dependent on blockade of Ca^{2+} influx to muscular cells. The isomer S(−)-bupivacaine is more potent and less toxic which could represent a valuable clinical advantage to use as broncholitic agent.

1. Introduction

Lidocaine has been used for preventing bronchospasm caused by mechanical activation during general anesthesia or bronchoscopy procedure. Inhalation of a combined formulation of lidocaine with salbutamol, a beta-2 adrenoceptor agonist markedly reduces the bronchospasm reaction during airway manipulation in anesthesia (Nakahara et al., 2000). Bupivacaine the most frequently local anesthetic used for regional anesthesia is approximately 5-fold more potent than lidocaine to block nerve conduction. Comparative analysis between bupivacaine and lidocaine to produce relaxation on airway smooth muscle is not available. Many mechanisms are proposed to explain the relaxant effect of lidocaine in airway smooth muscle. Lidocaine potentiates the relaxant response induced by drugs which increase formation of adenosine 3′,5′-cyclic monophosphate (cAMP) (Nakahara et al., 2000). Moreover, lidocaine increases the relaxant effect of atrial natriuretic peptide (ANP) in bovine tracheal smooth muscle preparations pre-contracted with methacholine. This effect is consequent to an inhibition of M_2 muscarinic receptor-mediated signal transduction (Yunoki et al., 2003a,b). Other studies have shown that lidocaine slightly inhibited the influx of Ca^{2+} on potassium- and ACh-induced contraction, which could contribute to the relaxation of the airway smooth muscle (Kai et al., 1993; Nosaka et al., 1989).

The airway epithelial cells modulate the baseline tone and reactivity of the underlying smooth muscle by releasing epithelium-derived relaxing factors (EpDRFs) (Hashiba et al., 1999; Raeburn et al., 1986), such as nitric oxide (NO) (Buga et al., 1989; Nijkamp et al., 1993) and prostaglandins (PG) (Farmer et al., 1987; Tschirhart et al., 1987). Arachidonic acid inhibits carbachol (CCh) and histamine (HA) induced contractions on airway smooth muscle, but this relaxation is epithelium-dependent (Farmer et al., 1987). Pre-treatment with indomethacin inhibits the arachidonic acid-induced relaxation (Farmer et al., 1987). There is no information regarding the potency

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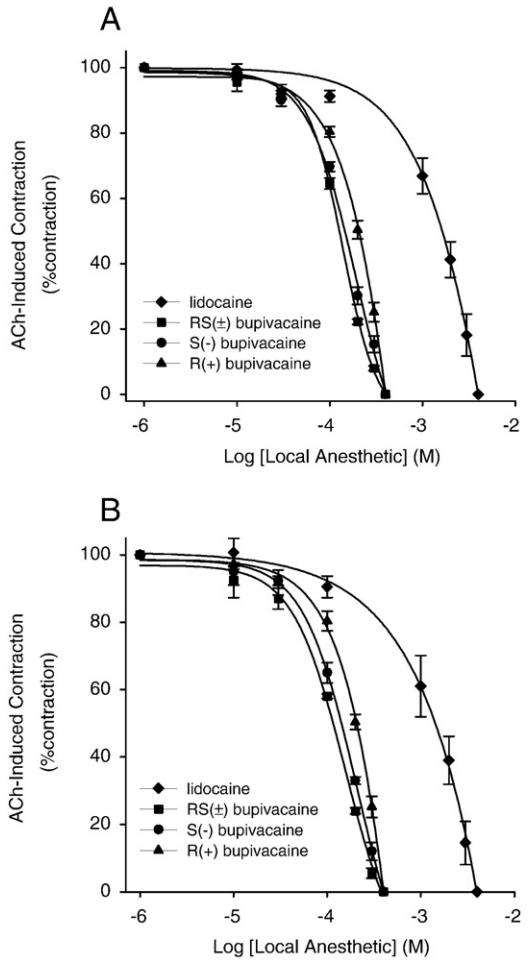

Fig. 1. Relaxant effect of lidocaine, RS(±)-, R(+)- and S(−)-bupivacaine on ACh-induced (10^{-5} M) contraction in rat airway smooth muscle with intact (A) and denuded (B) epithelium. Tension is expressed as percentage of the peak contraction response to ACh. The data represents mean ± S.E.M. ($n = 8$). * $P<0.01$ RS(±)-bupivacaine *vs* R(+)-bupivacaine, $^{\#}P<0.01$ RS(±)-, R(+)- or S(−)-bupivacaine *vs* lidocaine.

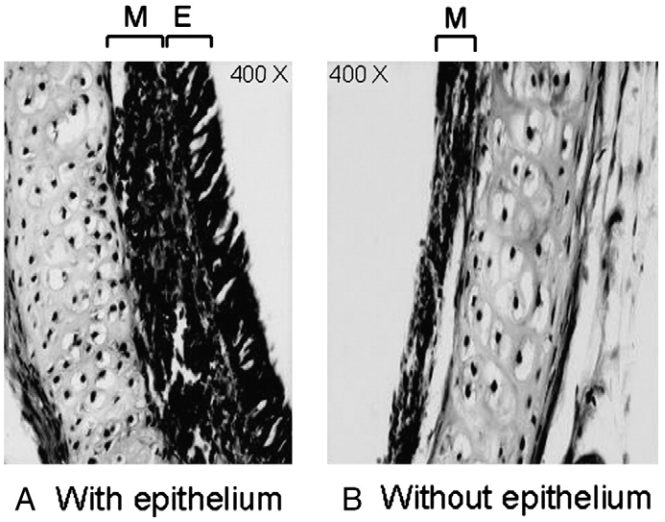

Fig. 2. Histopathology of transversal section of trachea fixed with formaldehyde and stained with hematoxylin and eosin. (A) Epithelium intact; (B) Epithelium denuded. M, smooth muscle and E, epithelium.

of bupivacaine and isomers to produce relaxation of airway smooth muscle in comparison to lidocaine. And also, possible mechanisms involved in the relaxation induced by these local anesthetics on this tissue were not yet established. Thus, the present work was designed to investigate the effects of bupivacaine and isomers on airways reactivity in rats, examining the possible mechanisms involved in it.

2. Materials and methods

2.1. Drugs

Racemic bupivacaine (RS(±)), S(−)-bupivacaine, R(+)-bupivacaine and lidocaine were gently donated by Cristalia Produtos Quimicos e Farmaceuticos Ltda (Itapira, SP, Brazil). Substances were dissolved in distilled water and prepared few minutes before use. Acetylcholine (ACh), N^G-nitro-L-arginine methyl ester (L-NAME), indomethacine and propranolol were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Isometric tension recording in tracheal rings

The Animal Care and Use Committee at the Universidade Federal do Rio de Janeiro approved the following protocols.

Male Wistar rats (220–300 g) were sacrificed by cervical dislocation under anesthesia with pentobarbital sodium (60 mg kg^{-1}) and the trachea was removed, cut in small rings (2–3 mm) and placed in vertical chambers (internal volume 10 ml) filled with Tyrode solution (in mM, NaCl 120; KCl 5.9; $MgCl_2$ 1.2; NaH_2P0_4 1.2; $NaHC0_3$ 18; $CaCl_2$ 2.5; glucose 11, pH 7.4) oxygenated with carbogen gas (95% O_2/5% CO_2) at 37 ± 0.2 °C. Each ring was attached to a force transducer (Grass mod. FT-03) which signal was conditioned by a Cyberamp (Axon Instruments, Inc.) and contractile response displayed and stored on a computer for future analysis using Axoscope software (Axon Instruments, Inc.). Preparations were stabilized for 2 h under 1 g resting tension, and the contractile response to acetylcholine (10 μM) was measured before and during exposure to increasing concentrations of lidocaine (0.01–2 mM) and bupivacaine and isomers (0.01–0.5 mM). At the end of each experiment, the integrity of epithelial cells was determined by the observation of trachea tissue stained with hematoxyline and eosin in optical microscope. In some experiments, the epithelium was mechanically removed to investigate the influence of functional epithelium to the relaxation induced by local anesthetics.

We investigated the relaxant effect of lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine in the presence of the cyclooxygenase type 1 and 2

Table 1
The 50% inhibitory concentration (IC_{50}) of local anesthetics on ACh-induced contraction in tracheal rings of rat.

Groups	Lidocaine	RS (±) bupivacaine	S (−) bupivacaine	R (+) bupivacaine
Epithelium intact	1.25 ± 0.014	0.11 ± 0.007[a]	0.15 ± 0.004[a]	0.19 ± 0.008[a,b]
Epithelium denuded	1.26 ± 0.002	0.12 ± 0.007[a]	0.15 ± 0.004[a]	0.20 ± 0.008[a,b]
Indomethacine	1.22 ± 0.012	0.13 ± 0.001[a]	0.14 ± 0.003[a]	0.20 ± 0.007[a,b]
L-Name	1.12 ± 0.015	0.09 ± 0.009[a]	0.14 ± 0.003[a]	0.17 ± 0.006[a,b]

The values (in mM) represents the mean ± S.E.M. ($n = 8$) of IC_{50} determined for each experiment calculated by equation $y = y_0 + a / (1 + e^{-x - x0/b})$ where $y_0 = y_{min}$; $a = y_{max} - y_{min}$; b = slope and $x_0 = IC_{50}$.

[a] $P<0.01$ RS(±)-, R(+)-, S(−)-bupivacaine *vs* lidocaine.

[b] $P<0.01$ R(+)-bupivacaine *vs* RS(±)-bupivacaine.

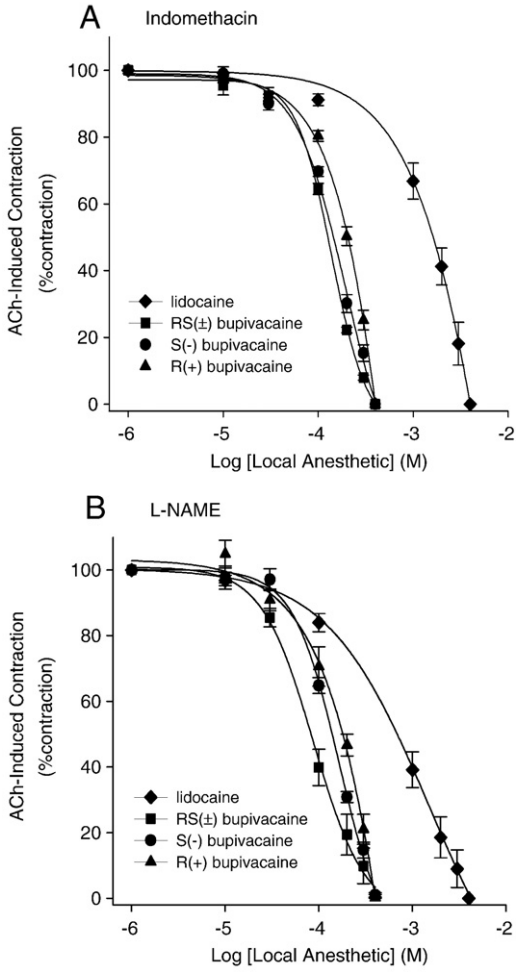

Fig. 3. Effect of pre-treatment with indomethacin (10^{-5} M) and L-NAME (10^{-4} M) on relaxation of ACh-induced (10^{-5} M) contraction by lidocaine (2 mM), RS(±)- (0.2 mM), R(+)- (0.2 mM) and S(−)-bupivacaine (0.2 mM) in rat airway smooth muscle. Each point represents mean ± S.E.M. ($n = 8$).

inhibitor, indomethacin. Tracheal rings were incubated with indomethacin (10 μM) for 30 min followed by exposure to acetylcholine (10 μM). After stabilization of the plateau phase of acetylcholine-induced contraction, increased concentrations of lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine were added to the preparations. Similar experimental protocol was performed to investigate the role of NO formation on the relaxation induced by local anesthetic. Pre-incubation of tracheal rings with L-NAME (100 μM), a NO synthase inhibitor was followed by exposure to acetylcholine (10 μM) and then, increased concentrations of local anesthetics.

To evaluate the importance of Ca^{2+}-influx on local anesthetic-induced relaxation we used a protocol previously described (Perusquía and Villalón, 1999). The trachea rings were exposed to a high KCl solution (40 mM) in the absence of Ca^{2+} (0-Ca^{2+}). The composition of the free-calcium solution was in mM: NaCl 84; KCl 40; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 18; and glucose 11. High potassium concentration produced a slight contraction of the trachea rings in some experiments and, upon its equilibrium, $CaCl_2$ (1.5 mM) was added to evoke a contraction. Calcium-contraction was repeated 3–4 times to eliminate run down of the process. Depolarized tracheal rings were incubated with each local anesthetic in the presence of 0-Ca^{2+} solution (K^++0-Ca^{2+}) during 10 min before the addition of $CaCl_2$ (1.5 mM). The amplitude of Ca^{2+}-induced contraction was compared between experiments performed in the presence or absence of pre-exposure to local anesthetics. At the end of each experiment, tracheal rings were exposed to $CaCl_2$ (1.5 mM) in the depolarizing solution without local anesthetic to observe the reversibility of the effects.

2.3. Statistical analysis

The relaxation induced by lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine was expressed as percentage of maximal tension. All data were expressed as mean ± S.E.M. The difference between the effect of different concentrations of local anesthetics was considered statistically significant when $P<0.05$, using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test. For the comparison of multiple groups, ANOVA was used followed by Newman Keuls test. The concentration-response curve was fitted by nonlinear regression

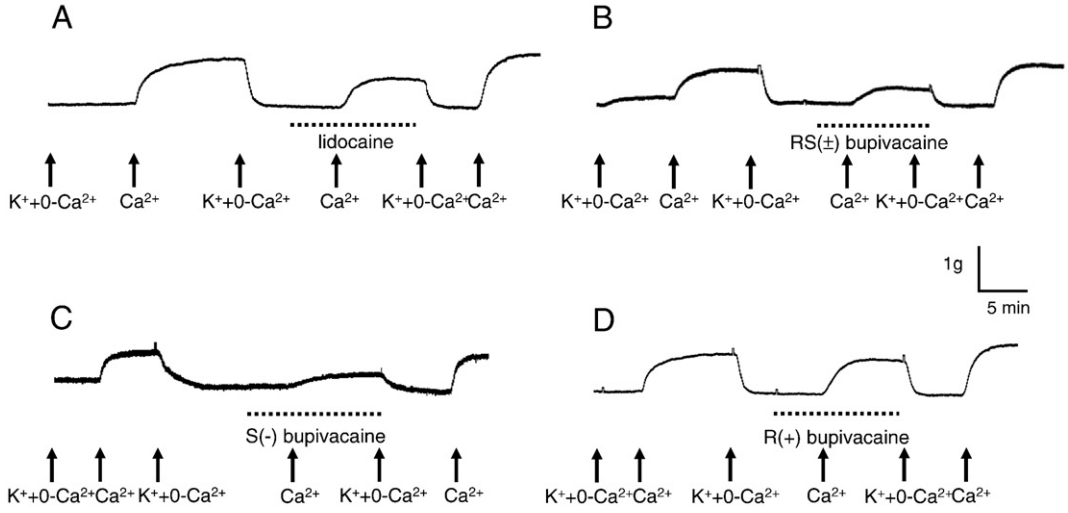

Fig. 4. Typical tracing showing the effect of lidocaine (2 mM) (A), RS(±)-bupivacaine (0.2 mM) (B), R(+)-bupivacaine (0.2 mM) (C) and S(−)-bupivacaine (0.2 mM) (D) on contractions induced by $CaCl_2$ (1.5 mM) in tracheal smooth muscle previously depolarized by KCl (40 mM) (K+0-Ca^{2+}). The dashed line represents the period of exposure of trachea rings to the indicated local anesthetics. The arrows represent the time in which the Tyrode solution was modified. Ca^{2+}, 1.5 mM of $CaCl_2$ added into solution; W, the trachea rings were washed with K-0-Ca^{2+} solution to cause relaxation of smooth muscle.

analysis for each experiment and the concentration of anesthetic that caused 50% of the inhibition (IC_{50}) of ACh-induced contraction was determined. The concentration–response curves were fitted to the equation $y = y_0 + a / (1 + e^{-x - x0/b})$ where y = percentage of isometric tension; $y_0 = y_{min}$; y_{min} = percentage of isometric tension at lowest drug concentration; y_{max} = percentage of isometric tension at highest drug concentration; $a = y_{max} - y_{min}$; b = slope and $x_0 = IC_{50}$.

3. Results

Lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine promoted relaxation of ACh-induced contraction in a concentration-dependent manner on tracheal smooth muscle with intact epithelium (Fig. 1A). Complete relaxation was observed with all local anesthetics at the highest concentration tested. Six and half to 11.3-fold differences in potency was observed between lidocaine and bupivacaine and its isomers. RS(±)-bupivacaine was the most potent among all tested local anesthetics (Table 1). The IC_{50} for lidocaine; RS(±)-; S(−)- and R(+)-bupivacaine to reduce the acetylcholine-induced contraction was 1.25 ± 0.01; 0.11 ± 0.01; 0.15 ± 0.01 and 0.19 ± 0.01 mM, respectively. RS(±)-bupivacaine was more potent than R(+)-bupivacaine ($P<0.01$) and similar to S(−)-bupivacaine (Table 1).

Mechanic removal of epithelium, confirmed by optical microscopic observation (Fig. 2), did not modify the potency or efficacy of local anesthetic to produce relaxation of acetylcholine-induced contraction in tracheal rings (Fig. 1B). As shown in Table 1, the IC_{50} for lidocaine; RS(±)-; S(−)- and R(+)-bupivacaine was 1.26 ± 0.01; 0.12 ± 0.01; 0.15 ± 0.01 and 0.20 ± 0.01 mM. Also, the IC_{50} for RS(±)-bupivacaine was significantly lower ($P<0.01$) than R(+)-bupivacaine but not for S(−)-bupivacaine.

Pre-incubation with indomethacin (10 μM) or L-NAME (100 μM) did not alter the relaxation on trachea smooth muscle induced by local anesthetics (Fig. 3A and B). As shown in Table 1, the IC_{50} for lidocaine; RS(±)-; S(−)- and R(+)-bupivacaine was not significantly different in the absence or presence of indomethacin and L-NAME (Table 1).

Typical recording to demonstrate the effect of local anesthetics on Ca^{2+} influx in tracheal rings is shown in the Fig. 4. Replacement of saline to K^++0-Ca^{2+} solution promoted a slight contraction in some experiments (Fig. 4B). Addition of $CaCl_2$ (1.5 mM) to preparation caused a rapid and sustained contraction, which was totally reversed when Ca^{2+} was washed out. Exposure to lidocaine (2 mM) (Fig. 4A), RS(±) (0.2 mM) (Fig. 4B), R(+) (0.2 mM) (Fig. 4C) or S(−)-bupivacaine (0.2 mM) (Fig. 4D) reduced the amplitude of Ca^{2+}-induced contraction. The concentration of lidocaine was 10-fold higher than the others anesthetics to reduce the Ca^{2+}-induced contraction. Fig. 5 shows that the inhibition of contraction induced by voltage-dependent Ca^{2+} channel activation by S(−)-bupivacaine (78.8 ± 7.4%) is significantly greater ($P<0.01$) than R(+)-bupivacaine (25.6 ± 9.5%) and RS(±)-bupivacaine (41.8 ± 6.7%). Also, the inhibition of lidocaine (59.4 ± 7.7%) was more intense than of R(+)-bupivacaine.

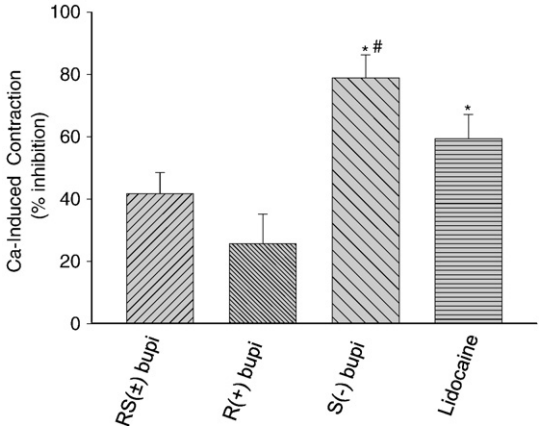

Fig. 5. Percent of inhibition induced by RS(±)-bupivacaine (RS(±)-bupi) (0.2 mM), R(+)- bupivacaine (R(+)-bupi) (0.2 mM), S(−)-bupivacaine (S(−)-bupi) (2 mM) and lidocaine (Lido) (0.2 mM) on Ca^{2+}-induced contractions on tracheal smooth muscle of rat. Before adding $CaCl_2$ (1.5 mM) the muscle was exposed to a depolarizing solution (K+0-Ca^{2+}) as described in the Materials and methods section. The bars represent mean ± S.E.M. ($n=6$). * $P<0.01$ S(−)-bupivacaine and lidocaine *vs* R(+)-bupivacaine, # $P<0.01$ S(−)-bupivacaine *vs* RS(±)-bupivacaine.

4. Discussion

The present work demonstrated that bupivacaine and its isomers produce relaxation of tracheal smooth muscle which contraction was induced by acetylcholine. The potency of these local anesthetics was greater than lidocaine, the unique local anesthetic clinically used for treatment of bronchospasm The potency of RS(±)-, S(−)- and R(+)-bupivacaine was 11.3 to 6.5-fold higher than lidocaine to promote relaxation on the airways muscle suggesting greater sensitivity of bupivacaine when compared to lidocaine. The relaxation of acetylcholine-induced contraction was not differently affected by S(−)- and R(+)-bupivacaine isomers, however, RS(±)-bupivacaine was more potent than R(+)-bupivacaine but not to S(−)-bupivacaine. The reason of absence of stereoselectivity in inducing relaxation of acetylcholine-induced contraction is already unknown, but could be related to multiple mechanisms since activation of M2 muscarinic subtype receptor by acetylcholine could activate different intracellular messengers involved in the contraction/relaxation of tracheal smooth muscle. The concentration of lidocaine necessary to induce neuronal blockade is also about 5-fold higher than for RS(±)-bupivacaine (Yano et al., 2006). Differences in potency among bupivacaine and its isomers have been demonstrated in sciatic nerve from rat. The concentration of RS(±)-bupivacaine to inhibit by 50% the conduction of motor nerve fibers was 2.5–3-fold lower than the isolated isomers (Trachez et al., 2005). Similar results were observed in the airway smooth muscle.

The airways epithelium layer plays an important role in modulating the basal tone and reactivity of smooth muscle (Butler et al., 1987; Murlas, 1986). In the airways, the muscle tone is modified by a balance between synthesis and release of epithelium-derived relaxing factors such as NO (Buga et al., 1989; Nijkamp et al., 1993) and prostaglandin (Farmer et al., 1987; Tschirhart et al., 1987). The importance of epithelium integrity to bupivacaine-induced relaxation was evaluated in experiments in which epithelial cells were mechanically removed from the trachea. Removal of epithelium cells significantly ($P<0.05$) increased the acetylcholine-induced contraction from 0.70 ± 0.11 ($n=6$) to 1.02 ± 0.22 g ($n=6$). The relaxant effect of lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine on the rat tracheal smooth muscle was not reversed by pre-treatment with indomethacin and L-NAME indicating that the action of local anesthetics did not depend on prostaglandin or NO release from epithelial cells of respiratory airway. These results confirmed that mechanical removal of epithelium did not modify the local anesthetics effect. Thus, we considered that lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine promoted relaxation of acetylcholine-induced contraction of tracheal airways by direct interaction to smooth muscle.

Recently, it has been demonstrated that lidocaine increases the relaxant effect of salbutamol and forskolin through the increase of cAMP (Nakahara et al., 2000). In the tracheal smooth muscle, the interaction of cholinomimetics such as acetylcholine and methacholine to muscarinic receptor type 2 (M2) reduces the intracellular concentration of cAMP (Sankary et al., 1988; Schaefer et al., 1995). There is evidence that lidocaine increases the relaxant effects of drugs that increases the cAMP accumulation and reduces the contraction mediated by muscarinic receptor agonists (Nakahara et al., 2000). The

mechanism of lidocaine in the smooth muscle is not well elucidated but there are evidences that correlate its effect of the airways to the blockade of M2 muscarinic receptors (Yunoki et al., 2003a,b).

It has previously demonstrated that lidocaine inhibits Ca^{2+} influx and reduces the sensitivity of contractile proteins in isolated tracheal rings, resulting in an attenuation of acetylcholine-induced contraction (Kai et al., 1993). In addition, local anesthetics blocked approximately 40% of myosin light chain kinase (MLCK) activity (Nosaka et al., 1989). Although tested in a single concentration, in the present study all local anesthetics inhibited partially the contraction induced by Ca^{2+} in depolarized preparation. Interestingly, intense blockade was observed with S(−)-bupivacaine suggesting an absence of correlation of this effect with the potency as local anesthetics because RS(±)-bupivacaine is the most potent to block neuron conduction. These data indicated an isomer-selective action and that an additional mechanism is involved in the local anesthetic induced-relaxation in airways.

In our experimental conditions, RS(±)-bupivacaine and its isomers promoted relaxation of acetylcholine-induced contraction on rat tracheal smooth muscle in a concentration-dependent manner. However, a concentration-dependent dual effect has been reported in guinea pig tracheal smooth muscle. Low concentration of RS(±)-bupivacaine ($6.9–55\times10^{-6}$ M) induced contraction and high concentration ($1.1–18\times10^{-4}$ M) relaxation (Lee et al., 1997). The dual effect of RS(±)-bupivacaine has been attributed to the S(−) but not to R(+)-isomer. The implication of S(−)-isomer for the dual action of local anesthetics has been also demonstrated in vascular smooth muscle (Tokinaga et al., 2007). The mechanism of bupivacaine-induced contraction at low concentration is not completely understood. However, there is evidence of increased intracellular Ca^{2+} concentration due to Ca 2+ release from intracellular store through the activation of Ca^{2+} release channel in vascular smooth muscle (Harnett and Biancani, 2003) and skeletal and cardiac cells (Komai and Lokuta, 1999). The mechanism of relaxation also is not clear but is probably related to inhibition of Ca^{2+} influx via L-type Ca^{2+} channel demonstrated indirectly in this study and in the isolated cardiac myocytes (Zapata-Sudo et al., 2001).

The epithelium-independent relaxant property of lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine could be advantageous for patients with abnormal airway hyperreactivity. There is evidence from human isolated tracheal muscle that epithelium removal increases the reactivity to methacholine (Raeburn et al., 1986). Clinical studies have indicated that lidocaine is a powerful broncho-spasmolytic agent (Groeben et al., 2000). This was confirmed in a randomized double-blinded study of 15 people in which lidocaine attenuated histamine-induced bronchospasm (Groeben et al., 1996; Groeben et al., 1998). Moreover, it has been shown that lidocaine augmented the spasmolytic effect of salbutamol in patients with bronchial hyperreactivity (Groeben et al., 1999; Groeben et al., 1998). Our study using an in vitro model shows that the IC_{50} for lidocaine, RS(±)-, S(−)- and R(+)-bupivacaine to promote relaxation of ACh-induced contraction was 0.03, 0.003, 0.004 and 0.005%, respectively. The concentration of lidocaine available for topic application in the airways is 2–4% indicating that it is possible that lower concentration of lidocaine could be used clinically to reduce bronchospasm. Bupivacaine and its isomers displayed similar cardiotoxicity (Zapata-Sudo et al., 2001). Clinically, RS(±)-bupivacaine shows moderate levels of cardiotoxicity which is attributed to the isomer R(+)-. Isomer S(−)- is less cardiotoxic and had a more intense spasmolytic effect than RS(±)- or R(+)-. Thus, S(−)-bupivacaine may provide an effective and safer alternative to lidocaine and/or RS(±)-bupivacaine in the treatment of broncospasm.

In conclusion, the potency of RS(±)-, S(−)- and R(+)-bupivacaine to produce relaxation of acetylcholine-induced contraction is 6.5 to 11.3-fold higher than lidocaine and this effect is not dependent on the release of epithelium relaxant factors. Their action is stereoselective and partially dependent on blockade of Ca^{2+} influx to muscular cells. The isomer S(−)-bupivacaine is more potent and less toxic which could represent a valuable clinical advantage to use as broncholitic agent.

Acknowledgments

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