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Effects of Acetazolamide and 4-Aminopyridine on CO_2-induced Slowly Adapting Pulmonary Stretch Receptor Inhibition in Rats

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Abstract

Inhibitory responses of slowly adapting pulmonary stretch receptor (SAR) activity to CO_2 inhalation (maximal tracheal CO_2 concentration ranging from 9.5 to 12.5%) for ~60 s were examined before and after administration of acetazolamide (a carbonic anhydrase inhibitor) or 4-aminopyridine (4-AP, a K^+ channel blocker). The experiments were performed in 35 anesthetized, artifically ventilated rats after unilateral vagotomy. Sixty-eight of eighty-four SARs were inhibited by CO_2 inhalation. The SAR inhibition was attenuated by pretreatment with either acetazolamide (20 mg/kg, $n = 10$) or 4-AP (0.7 and 2.0 mg/kg, $n = 10$). In other series of experiments, stainings to show the existence of carbonic anhydrase (CA) enzymatic reaction were not found in the smooth muscle of either extrapulmonary or intrapulmonary bronchi. Protein gene product 9.5 (PGP 9.5)-immunoreactive SAR terminals to form leaflike extensions were found in the bronchioles at different diameters and were smooth-muscle-related receptors. But in the same sections, CA isozyme II-like (erythrocyte CA) immunoreactive SAR terminals were not identified. These results suggest that CO_2-induced inhibition of SARs may be involved in the CA-dependent CO_2 hydration in addition to the activation of 4-AP sensitive K^+ currents.

Key words: carbonic anhydrase inhibitor, CO_2 inhalation, enzymatic reaction, immunohistochemical examination, potassium channel blocker, slowly adapting pulmonary stretch receptor

Introduction

The inhibition of slowly adapting pulmonary stretch receptor (SAR) activity occurs during inhalation of CO_2 gas mixtures and this inhibition is not accompanied by the change in lung mechanics (Sant' Ambrogio *et al.*, 1974; Coleridge *et al.*, 1978; Matsumoto *et al.*, 1996). Indeed, pretreatment with the bronchodilators does not significantly alter the inhibitory response of SAR activity to inhaled wood smoke (Lai and Kou, 1998) containing a high concentration (~15%) of CO_2 (Larson and Koenig, 1994; Kou *et al.*, 1995). The administration of a carbonic anhydrase (CA) inhibitor, acetazolamide, attenuates or blocks the inhibitory effects of CO_2 inhalation (Sant' Ambrogio *et al.*, 1974; Matsumoto *et al.*, 1996) and wood smoke delivery (Lai and Kou, 1998) on SAR activity. Because no CA activities are found in the airway smooth muscle of bronchi (Matsumoto *et al.*, 1996), it is possible that CO_2-induced SAR inhibition is mediated by an increase in the H^+ concentration at the receptor site, but the question arises whether CA enzymes that catalyze the reaction are localized in the nerve endings of SARs.

On the other hand, an increase in Cl^-or K^+ conductance, resulting in hyperpolarization of the neuronal membrane, is seen during a decrease in the extracellular pH produced by CO_2 in neurons of the marine mollusk *Aphysia californica* (Brown, 1972). A similar mechanism has recently been proposed by Matsumoto *et al.* (1999) to explain the inhibitory action of CO_2 on SAR activity because inhibition of SARs by CO_2 inhalation is attenuated by 4-aminopyridine (4-AP), one of the K^+ channel blockers, but not by the other K^+ channel blocker tetraethylammonium (TEA).

Nevertheless, there are no studies investigating the difference between the blocking actions of acetazolamide and 4-AP on the inhibitory response of SARs to CO_2 inhalation. To elucidate whether there is a close correlation between a CA-dependent CO_2 hydration or a generalized action of K^+

channels and the inhibition of SARs associated with CO_2 inhalation, we designed five different types of experiments. First, the responses of SARs to CO_2 inhalation were examined before and after administration of acetazolamide. Secondly, the responses of SARs to CO_2 inhalation before and after administration of 4-AP were examined. These two experiments were performed in anesthetized, artificially ventilated rats with unilateral vagotomy. Thirdly, we determined whether the staining of a CA enzymatic reaction within the smooth muscle of extra- pulmonary and intrapulmonary bronchi is due to a specific or nonspecific activity of the enzyme. Finally, we evaluated the presence of immunostaining for protein gene product 9.5 (PGP 9.5) capable of visualizing the contour of SAR terminals in the intrapulmonary bronchi or bronchiole and in the same sections we further determined whether the SAR terminals with a positive staining for PGP 9.5 also exhibit carbonic anhydrase isozyme II (CAII)-like immunoreactivity.

Materials and methods

Animal preparation

All experimental protocols used in this study were approved by the Animal Use and Care Committee of Nippon Dental University. Forty-three Wister rats, weighing 280–360 g, were anesthetized with i.p. injection of sodium pentobarbital (45–50 mg/kg). The trachea was exposed through a midline incision in the neck and cannulated below the larynx. In order to obtain a large space for liquid paraffin, the trachea and esophagus were dissected free and retracted rostrally. Tracheal pressure (P_T) was measured by connecting a polyethylene catheter inserted into the tracheal tube to a pressure transducer. The right carotid artery and jugular vein were cannulated for monitoring blood pressure (BP) and for injecting drugs or 0.9% NaCl solution, respectively. During the course of the experiments the level of anesthesia was monitored by assessing the pressor response to tail pinch and supplemental doses (9–10 mg/kg, i.v.) of sodium pentobarbital were administered to maintain the level of anesthesia, abolishing a prompt change in BP seen during the corneal reflex and pain reflex induced by tail pinch. Then the left vagus nerve was exposed and sectioned. The right vagus nerve was left intact. The rectal temperature was maintained at around 37°C by means of a heating lamp or pad. The animals were paralyzed with i.v. injection of gallamine (5–10 mg/kg) for 30–60 min and additional doses (3–5 mg/kg) of gallamine were administered to eliminate spontaneous respiratory movements as needed. The stroke volume of the respirator was set at 10 ml/kg and its frequency ranged from 50 to 60 cycles/min. CO_2 inhalation was produced by adding the gas mixtures supplying the respiratory pump. The gas mixtures were first adjusted via each valve attached to the flowmeters of O_2, CO_2 and N_2. During CO_2 inhalation, tracheal CO_2 concentration ranging at 9.5–12.5% and tracheal O_2 contration ranging at 25–30% were maintained by changing gas flow.

Measurement of SAR activity

The single unit activity of SARs was recorded by means of the same techniques as described in a previous study (Matsumoto *et al.*, 2000). Briefly, fine afferent nerve fibers were split from the desheathed never trunk of the left vagus nerve, placed on a unipolar silver electrode and submerged in a pool with warm liquid paraffin (37–38°C). The SARs were identified, on the basis of their firing behavior during inflation, as follows: (i) the SAR increased their discharge during inflation and decreased their activity during deflation; (ii) the increase in SAR activity was proportional to the increase in the inflation volume of the respirator; and (iii) the discharge of SARs continued when occlusion of the tracheal tube during inspiration kept the lung in an inflated state. The SAR activity was amplified and selected by means of a window discriminator for counting the number of impulses.

Experimental design

A total of 84 SARs were recorded from 35 rats to examine their response to CO_2 inhalation. In 20 SARs recorded from 20 rats, SAR responses to CO_2 inhalation were compared before and after administration of acetazolamide (a CA inhibitor, $n = 10$) and 4-AP (a K^+ channel blocker, $n = 10$). The experiments were designed to compare the roles of a CA and a generalized K^+ channel in the inhibitory responses of SAR activity to CO_2 inhalation.

Drugs

Acetazolamide (500 mg; Takeda Pharmaceutical Corporation, Japan) was diluted with 0.9% NaCl (20 mg/ml). 4-AP (20 mg; Sigma Chemical Co., St Louis, MO) was dissolved and diluted with 0.9% NaCl solution.

Statistical analysis

During control conditions, firing rates of the SARs during one whole respiratory cycle were measured over several respiratory cycles and expressed as impulses/second (imp/s). The SAR responses to CO_2 inhalation for 60 s (tracheal CO_2 concentration ranging from 9.5 to 12.5%) were obtained by counting the firing rates of receptors at 10 s intervals and by performing the measurements over 120 s and the average activities during one whole respiratory cycle were expressed as imp/s. Similarly, control values for P_T and responses of P_T to CO_2 inhalation were measured. The statistical significance of the time-dependent effects of acetazolamide and 4-AP on the responses of SAR activity and P_T to CO_2 inhalation was first calculated by a two-way analysis of variance (ANOVA) for repeated measurements. The effects of acetazolamide and 4-AP on the responses of SAR activity to CO_2 inhalation were also analyzed by means of the modified *t*-statistics and further assessed by Bonferroni's test for one

comparison ($k = 1$) to the control. In addition, the decreases in baseline SAR activity during one whole respiratory cycle induced by CO_2 inhalation in the absence and presence of acetazolamide (20 mg/kg) and 4-AP (0.7 and 2.0 mg/kg) were analyzed by a paired *t*-test. Furthermore, statistical comparisons of acetazolamide and 4-AP treatments on the maximal inhibition of SAR response to CO_2 inhalation were made by a two-sample *t*-test with Welch's correction. All values were expressed as means ± SE. A *P*-value <0.05 was considered statistically significant.

Examination of an enzymatic reation of CA in the airway smooth muscle

In three rats, under artificial ventilation the chest was widely opened at the midline. The animals were transcardially perfused with a fixative, consisting of 4% paraformaldehyde 0.1 M phosphate-buffered saline (pH = 7.4), after perfusion with 0.9% NaCl solution (500 ml). The trachea and lung tissues were removed immediately and air from the lungs was sucked out by a vacuum pump. The tissues were stored with the same solution for 1 h and then were immersed in 20% sucrose solution, embedded in ornithine carbamoyl transferase compound and frozen. Subsequently, they were serially sectioned at a thickness of 20 μm on a cryostat, and frozen sections were reacted in Hansson's solution for 8–13 min (Hansson, 1967; Sugai *et al.*, 1981). Finally, we determined the enzymatic reaction of CA in the smooth muscle located in both extra- and intrapulmonary bronchi. In order to examine a specific reaction of CA enzyme, frozen sections were first incubated by using the cobalt capture method of Hansson (1967) and then histological sections were incubated in Hansson's solution containing 10^{-5} M acetazolamide.

Examination of immunohistochemical activities of both PGP 9.5 and CA isozyme II in SAR nerve terminals in the smooth muscle layer of the bronchus

Three rats were used in immunofluorescent studies. Following the fixation, serial sections with a thickness of 20 μm were cut in a cryostat (Kryostat 1720, Leica). Cryostat sections were mounted onto APS-coated glass slides. Sections were washed in PBS for 5 min and endogenous peroxidase was inactivated by treatment with 3% H_2O_2. Non-specific immunoreactivity was inhibited by 5% skim milk in PBS for 20 min at room temperature. Sections were exposed overnight to sheep polyclonal antibody against CA isozyme II (1:1000; Biogenesis, Poole, UK) and rabbit polyclonal antibody against PGP 9.5 (1:5000; Biogenesis, UK), washed three times in 0.05% Tween 20 in PBS (5 min per time) and incubated for 3 h with secondary antibodies at room temperature. The fluorescently labeled secondary antibodies used were rhodamine-conjugated affinity-purified goat anti-rabbit IgG (1:100; Chemicon, CA) and fluorescent affinity-purified donkey anti-sheep IgG (1:100; Chemicon CA). After the sections were rinsed in PBS, immunofluorescence was visualized by using the appropriate filters. The location of immunofluorescent sections was confirmed by the serial sections without stainings.

Results

Effect of acetazolamide on response of SARs to CO_2 inhalation

All the SARs tested were classified into two different groups: 'low-threshold' receptors fired during both inflation and deflation (56/84) and 'high-threshold' receptors fired during inflation only (28/84). Of the 84 SARs tested, 68 were inhibited when inhalation of CO_2 gas mixtures (maximum tracheal CO_2 concentration ranging from 9.5 to 12.5%) was maintained for approximately 60 s, but the remainder SARs was unaffected. Typical examples of the effect of acetazolamide (20 mg/kg), a CA inhibitor, on the responses of SARs and P_T to CO_2 inhalation are shown in Figure 1. Before administration of acetazolamide, the inhibition of the SAR activity occurred immediately after the onset of increased tracheal CO_2 concentration. The SARs ceased their firing at ~15 s after CO_2 inhalation and such an effect lasted for the period of remaining CO_2 inhalation. The response was not associated with any significant change in P_T. After CO_2 inhalation ceased, SARs did not return to the control activity within 60 s (Figure 1A). The inhibitory effect of CO_2 inhalation was partly diminished by pretreatment with acetazolamide (20 mg/kg), which elicited hypocapnia and had no significant effect on P_T (Figure 1B). The responses of 10 different SARs in 10 rats to CO_2 inhalation were compared before and after pretreatment with acetazolamide (20 mg/kg) (Figure 2). At 10 s after CO_2 inhalation, the SAR activity during one whole respiratory cycle was decreased from 23.9 ± 1.9 to 9.3 ± 2.6 imp/s. The decrease in SAR activity became more prominent at 20 s after CO_2 inhalation. The average discharges of SARs in acetazolamide (20 mg/kg)-treated animals were 24.8 ± 2.2 imp/s and did not show any significant change as compared to those in the control. The inhibitory effect of CO_2 inhalation on SAR activity was significantly suppressed by pretreatment with acetazolamide. CO_2 inhalation before and after pretreatment with acetazolamide had no significant effect on P_T (Figure 2). The administration of acetazolamide did not cause any significant change in BP. The mean BP (MBP) values during control and after acetazolamide treatment were 86.7 ± 2.9 and 87.5 ± 3.1 mm Hg, respectively and 98.2 ± 3.6 and 99.4 ± 3.7 mm Hg after CO_2 inhalation, respectively. The maximal changes in MBP in response to CO_2 inhalation were not significantly altered by acetazolamide treatment.

Effect of 4-AP on response of SARs to CO_2 inhalation

The typical effect of 4-AP (2.0 mg/kg), a K^+ channel blocker, on the responses of SAR activity and P_T to CO_2 inhalation are shown in Figure 3A,B. The inhibitory action of CO_2 on

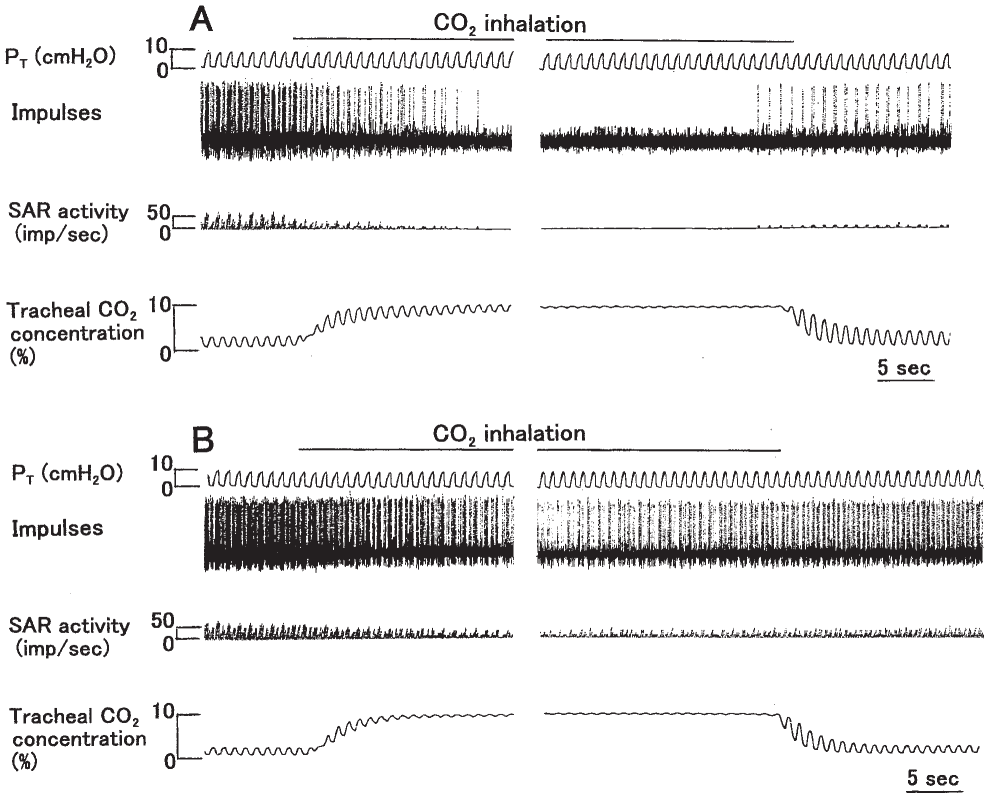

Figure 1 Effect of acetazolamide on the response of PT and SAR activity to CO_2 inhalation. **(A)** Control. **(B)** After i.v. administration of acetazolamide (20 mg/kg). Straight line, period of increased tracheal CO_2 concentration.

SAR activity was greatly diminished by pretreatment with 4-AP (2.0 mg/kg), which produced a significant increase in the SAR activity during both inflation and deflation and had no significant effect on P_T. We compared the responses of 10 different SARs in 10 rats to CO_2 inhalation before and after pretreatment with 4-AP at 0.7 and 2.0 mg/kg (Figure 4). The average discharges of SARs during one whole respiratory cycle were 20.5 ± 1.7 imp/s in the control and 35.5 ± 1.8 and 44.4 ± 1.7 imp/s in 4-AP-treated animals at 0.7 and 2.0 mg/kg, respectively. At 10 s after CO_2 inhalation the average discharges of SARs decreased to 6.4 ± 2.3 imp/s. The decrease in SAR activity had a more pronounced effect at 20 s after CO_2 inhalation. The K^+ channel blocker 4-AP (0.7 and 2.0 mg/kg) significantly reversed the inhibitory effect of CO_2 inhalation on SAR activity and this reversible effect was predominant in the case of 2.0 mg/kg 4-AP. The increase in BP occurred after the administration of 4-AP, but this pressor effect was transient. The MBP values were 85.2 ± 2.8, 86.4 ± 2.9 and 86.9 ± 3.1 mm Hg during control and after 4-AP treatment 0.7 and 2.0 mg/kg, respectively and 97.3 ± 3.4, 98.5 ± 3.6 and 99.8 ± 3.8 mm Hg during CO_2 inhalation, respectively. 4-AP treatment had no significant effect on the maximal change in MBP induced by CO_2 inhalation.

A comparison of acetazolamide and 4-AP effects on CO_2-induced SAR inhibition

All the SARs tested showed the maximal reduction in SAR activity at 60 s after CO_2 inhalation. On average, the maximal reductions in SAR activity produced by CO_2 inhalation were 83.9 ± 5.2 and 54.8 ± 3.4% of the baseline activity before and after acetazolamide (20 mg/kg, n = 10), respectively. Furthermore, the maximal reductions in SAR activity produced by CO_2 inhalation after 4-AP treatment at 0.7 mg/kg (52.4 ± 3.9% of the baseline activity, n = 10) and 2.0 mg/kg (13.1 ± 2.5% of baseline activity, n = 10) were different from the control response (before 4-AP treatment at 0.7 mg/kg, 83.1 ± 5.8%; before 4-AP treatment at 2.0 mg/kg, 82.6 ± 5.9%). When the acetazolamide- and 4-AP-induced suppressive effects on CO_2-induced SAR inhibition were evaluated together, the administration of 4-AP at 2.0 mg/kg significantly increased as compared to the acetazolamide (20 mg/kg) or 4-AP (0.7 mg/kg) administration (Figure 5).

Histochemical examination of CA activity in the airway smooth muscle

Histochemical examination of CA activity in the airway smooth muscle was performed using 32 sections. In 18 of the

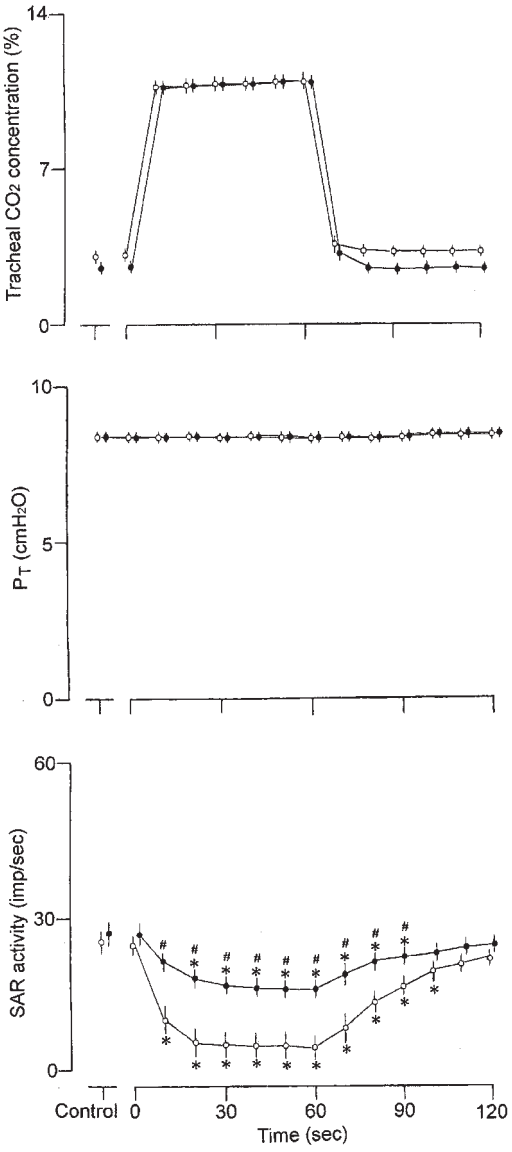

Figure 2 Mean data showing changes in PT and SAR activity in response to CO_2 inhalation before (open circles) and after (filled circles) intravenous administration of acetazolamide 20 mg/kg. 0, the onset of increased tracheal CO_2 concentration. *Significantly different ($P < 0.05$) from control values. Significantly different ($P < 0.05$) from acetazolamide effects. Values are means ± SE ($n = 10$).

32 sections, the CA activity was not found in the smooth muscle in either the extrapulmonary bronchi located below the carina (Figure 6A, 9/32) or the intrapulmonary bronchi (Figure 6B, 9/32), but the alveolar epithelial cells exhibited CA activity (Figure 6B). To determine whether the positive staining was specific for CA activity, we stained seven sets of serial sections (14 sections) in the absence and presence of acetazolamide (10^{-5} M). As show in Figure 6C,D, the staining of lung epithelial cells disappeared after inhibition of the CA activity with acetazolamide. Under these conditions, the smooth muscle in intrapulmonary bronchi did not show any specific reaction of the CA enzyme.

Immunoreactivities of PGP 9.5 and CA isozyme II in SAR nerve terminals

Based on evidence that the rabbit SAR structures have multiple endings, forming knoblike or leaflike extensions (Yu *et al.*, 2003), we could identify 6 SAR terminals immunoreactive for PGP 9.5 (red) in the bronchioles at different diameters. The main axon diameters of SAR terminal endings were measured the largest value from the axon bronches in the images. A typical example of the SAR structure is shown in Figure 7. This receptor was found in the bronchiole wall corresponding with a terminal bronchiole with a diameter of 100 μm and the main axon diameters of the receptors ranged from 1.2 to 1.8 μm (Figure 7A–D). As shown in Figure 7E, the leaflike terminal endings are buried in the wall associated with smooth muscle bands (Figure 7G). But in the same section, the CA-isozyme-II-like immunoreactive (green) SAR structures were not found (Figure 7B,C) and SAR terminal endings that did not show double-immunoreactive stainings (yellow) for PGP 9.5 and CA isozyme IIwere not identified (Figure 7F). Furthermore, similar receptor structures were observed in airways 144–167 μm in diameter and these receptors were embedded in the smooth muscle. The main axon diameters of these SARs ranged from 1.3 to 2.2 μm. The knoblike SAR terminal endings were buried in the bronchial wall, involving the smooth muscle layer (data did not show).

Discussion

The present study provided evidence that pretreatment with either acetazolamide or 4-AP significantly attenuated the inhibitory response of SAR activity to CO_2 inhalation (9.5–12.5%) for ~60 s. The expression of membrane-associated CA isozyne IV is known to be identified at the luminal side of the capillarly endothelium in the rat lung (Fleming *et al.*, 1993), but in this study the expression of CAII-like (erthrocyte CA) immunoreactive SAR structures was not identified in the another serial sections with positive immunoreactivity of PGP 9.5. When considering these results, taken together, we cannot completely rule out the possibity that an elevated PCO_2 may contribute to an inhibitory effect of SAR activity by increasing H^+ ion concentration at the receptor location.

The majority of SARs examined (~81%) were promptly inhibited when CO_2 gas mixtures were applied to the lungs and the rapid reaction of SARs occurred after CO_2 inhalation. The latter effect basically agrees with the fact that CO_2-induced SAR inhibition occurs even when the change in the airway CO_2 is isolated from that in the blood CO_2 (Sant' Ambrogio *et al.*, 1974; Bradley *et al.*, 1976; Coleridge *et al.*, 1978). Furthermore, a recent study has shown that the inhibition of SAR activity in the rat becomes apparent within one or two breaths when 6 ml of wood smoke, containing a high concentration of CO_2 (Larson and Koenig, 1994; Kou *et al.*, 1995), is delivered to the lungs and this inhibition is more prominent in deflation than inflation (Lai and Kou, 1998). The different actions of CO_2 on the

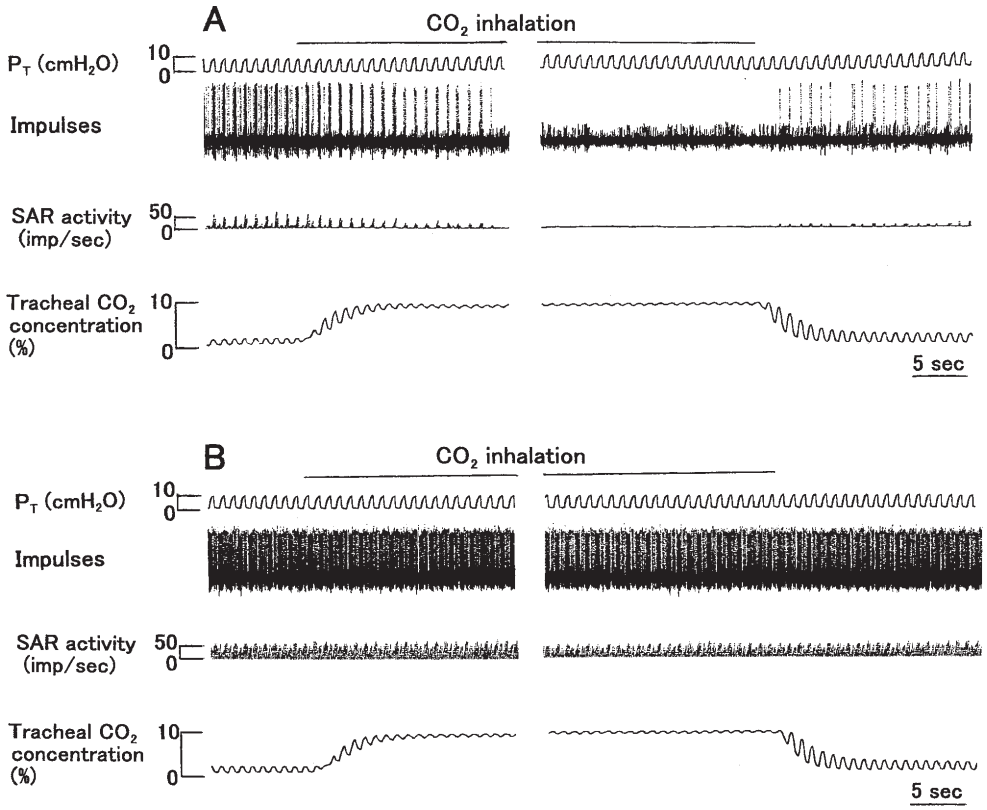

Figure 3 Effect of 4-AP on the responses of PT and SAR activity to CO_2 inhalation. **(A)** Control. **(B)** After intravenous administration of 4-AP (2.0 mg/kg). Straight line, period of increased tracheal CO_2 concentration.

SAR activity between inflation and deflation phases have been demonstrated by many investigators (Coleridge *et al.*, 1978; Matsumoto *et al.*, 1996, 1999) and this difference may be explained by the fact that the inhibitory effect of CO_2 on SAR activity becomes weaker at the higher transmural pressures (Mustafa and Purves, 1972; Bradley *et al.*, 1976).

Three different mechanisms for CO_2-induced SAR inhibition have so far been proposed (Schelegle and Green, 2001; Schelegle, 2003): (i) an indirect effect of CO_2 on the changes in bronchial smooth muscle tone (Nilsestuem *et al.*, 1979; Mitchell *et al.*, 1980; Ravi, 1986); (ii) a generalized pH effect on neuronal function (Coleridge *et al.*, 1978; Matsumoto *et al.*, 1996; Lai and Kou, 1998); and (iii) a direct effect of CO_2 on the change in SAR endings via activation of 4-AP-sensitive K^+ channels (Matsumoto *et al.*, 1999).

In this study, no detectable changes in P_T were found when CO_2 inhalation attenuated SAR activity in rats with intact innervation of the right vagus nerve. This finding resembled the observations demonstrating that CO_2-induced SAR inhibition was not significantly altered by pretreatment with the bronchodilators (Sant' Ambrogio *et al.*, 1974; Coleridge *et al.*, 1978; Lai and Kou, 1998). The sensitivity of SARs to CO_2 depends on their location in the trachobronchial tree (Sant' Ambrogio *et al.*, 1974; Bradley *et al.*, 1976) because SARs localized in extrapulmonary airways did not respond to CO_2 (Sant' Ambrogio *et al.*, 1974; Bartlett and Sant' Ambrogio, 1976; Lai and Kou, 1998).

Concerning the difference between CO_2 sensitivity in intrapulmonary and extrapulmonary bronchi, Pack (1981) postulated different concentrations of CA activity in the walls of different bronchi. However, no significant stainings of the CA enzymatic reaction were found in the smooth muscle of extrapulmonary and intrapulmonary bronchi of the rat. Similarity to the absence of CA enzymatic activity in airway smooth muscles has been reported in the rabbit (Matsumoto *et al.*, 1996). In a previous study on rabbits, acetazolamide treatment (20 mg/kg) could suppress CO_2-induced SAR inhibition (Matsumoto *et al.*, 1996).

In this study, pretreatment with acetazolamide at a dose of 20 mg/kg, which could cause the blockade of CA-dependent CO_2 hydration, attenuated CO_2-induced SAR inhibition, but the inhibitory effect of acetazolamide was significantly less than that of 4-AP. Although the exact mechanism by which an increase in the H^+ concentration results in a reduction in the SAR activity remains obscure, we found that 4-AP treatment strongly attenuated CO_2-induced SAR inhibition. A similar phenomenon has been demonstrated in the rabbit by Matsumoto *et al.* (1999), who suggested that an increase in the H^+ concentration at the receptor site may cause activation of the fast transient outward K^+ current (I_A) of the SAR terminals, which is sensitive to 4-AP.

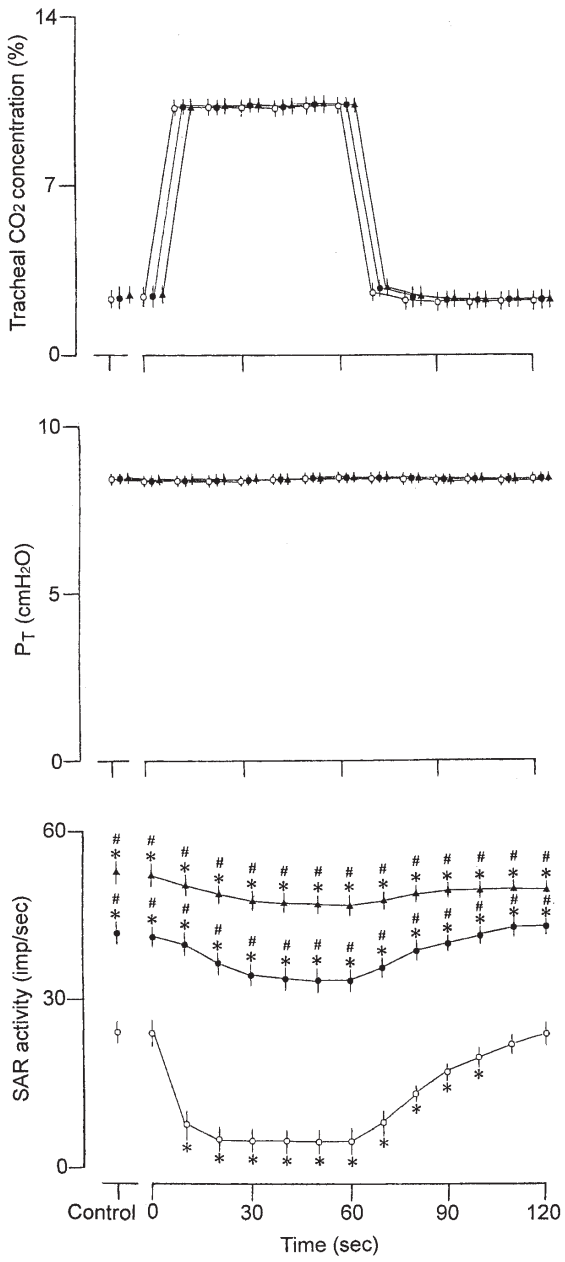

Figure 4 Mean data showing changes in PT and SAR activity in response to CO_2 inhalation before (open circles) and after intravenous administration of 4-AP at 0.7 mg/kg (filled circles) and 2.0 mg/kg (filled triangles). 0, the onset of increased tracheal CO_2 concentration. *Significantly different ($P < 0.05$) from control values. Significantly different ($P < 0.05$) from 4-AP effects. Values are means ± SE ($n = 10$).

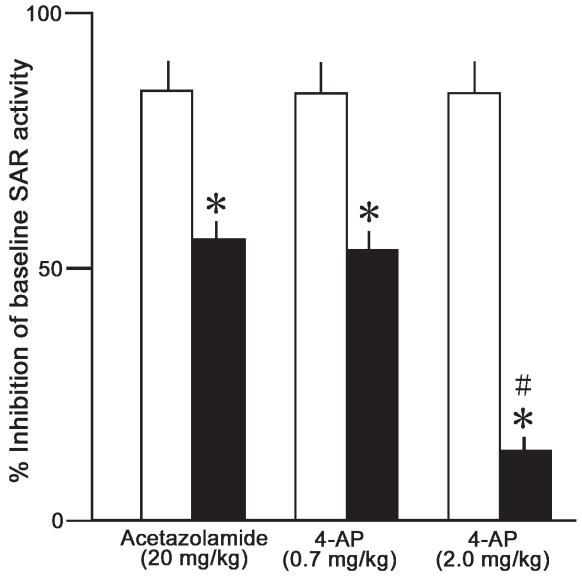

Figure 5 Group data for inhibitory responses of SAR activity to CO_2 inhalation before (open squares) and after (filled squares) pretreatment with acetazolamide and 4-AP. The inhibition was expressed as a percentage change from the baseline SAR activity. *Significantly different from control group. Significantly different from acetazolamide-treated group. Values are means ± SE ($n = 10$).

Indeed, we found that administration of 4-AP (2.0 mg/kg) increased the baseline activity of SARs. This finding is in agreement with the observation that the blocking action of 4-AP-sensitive K^+ channels leads to an increase in the excitability of SARs, as reported in a previous study on rabbits (Matsumoto *et al.*, 1999). The appearance of this effect is therefore related to the depolarization of the resting membrane potential and the repetitive firing seen in squid axons (Yeh *et al.*, 1976a,b).

The modulation of K^+ conductances can influence the shaping of action potentials, neuronal repetitive firing patterns and the summation of synaptic inputs in neurons (McLarnon, 1995). Based on the electrophysiological and pharmacological properties, several different types of K^+ channels have been identified; the most widely distributed K^+ currents are the *Shaker* (Kv 1), *Shab* (Kv 2), *Shaw* (Kv 3), *Shal* (Kv 4) (Butler *et al.*, 1989; Wei *et al.*, 1990) and a calcium-activated K^+ channel current (I_{KCa}) (Meech and Standen, 1975). Both I_A (*Shaker* and *Shal*) and I_K (a sustained K^+ current of delayed rectifier type, *Shab* and *Shaw*) regulate the timing of action potential formation and the repetitive firing pattern of neuronal cells (Dekin and Getting, 1987; Spigelmann *et al.*, 1992).

In the somatic membrane of pyramidal neurons, two major voltaged-gated K^+ currents, such as I_A and I_K, are identified: I_A is blocked by 4-AP, but not by the other K^+ channel blocker tetraethylammonium (TEA), whereas I_K is blocked by TEA, but not by 4-AP (Hoffman *et al.*, 1997; Bekkers, 2000). Similarly, in the myelinated axons of the rat sciatic nerve, 4-AP-sensitive K^+ channels are related to action potential repolarization (Kocsis *et al.*, 1987). Concerning the involvement of I_K on CO_2-induced SAR inhibition in the rabbit, pretreatment with TEA (2.0 and 6.0 mg/kg) had no significant effect on that response (Matsumoto *et al.*, 1999). Furthermore, the excitatory responses of deflationary SAR activity to lung deflation

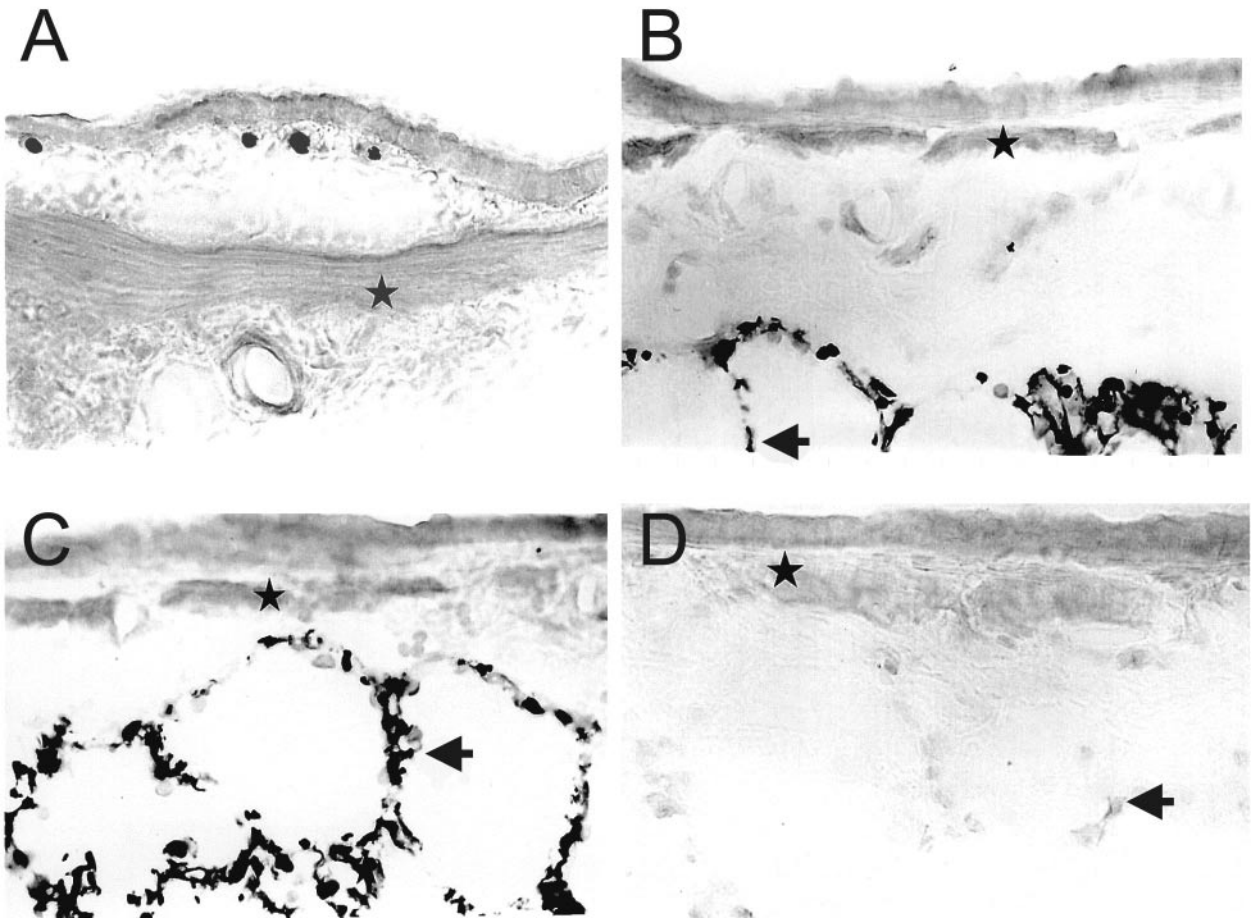

Figure 6 **(A)** Light micrograph of the extrapulmonary airways for CA activity. **(B)** Light micrograph of the intrapulmonary airways for CA activity. Note that lung epithelial cells (arrow) show intense staining but smooth muscles (star) do not show staining. Scale bars = 50 μm. **(C)** Light micrograph of the intrapulmonary airways for CA activity. Note that lung epithelial cells (arrow) show intense staining but smooth muscles (star) do not show staining. **(D)** Serial section of panel A, incubated with 10^{-5} M acetazolamide, an inhibitor of CA. Note that lung eipithelial cells (arrow) are CA-negative. Scale bars = 50 μm.

ranging from ~–15 to –20 cm H_2O were not significantly altered by administration of TEA (2.0 and 6.0 mg/kg) in the rat (Matsumoto *et al.*, 2002). According to McAlexander and Undem (2000), they demonstrated that nodose ganglion-derived airway sensory fibers (A δ range) in the guinea pig are relatively insensitive to TEA application (10 mM). It is therefore possible that changes in I_K after administration of either acetazolamide or 4-AP do not contribute to the inhibitory mechanism on the response of SAR activity to CO_2 inhalation. The 4-AP application is known to elicit a broad spike of action potentials (Kocsis *et al.*, 1987; Poulter and Padjen, 1995), but we could not identify such an effect because of measuring extracellular action potentials. In neurons of the marine mollusk *Aphysia california*, a decrease in extracellular pH induced by CO_2 causes hyperpolarization in accordance with increases in K^+ or Cl^- conductance (Brown, 1972). Presumably, the difference between the blocking actions of acetazolamide and 4-AP on CO_2-induced SAR inhibition may involve the difference of effects to prevent hyperpolarization of the membrane potential of the SAR terminals. This is incompatible with the observation proposing that acetazolamide treatment may cause alkalinity either in the receptors or in the tissue surrounding the receptor (Coates *et al.*, 1996).

Based on evidence that the laryngeal receptors are located beneath the airway epithelium or that histochemical localization of CA is found in surface epithelial cells (Wang *et al.*, 1994), CO_2 sensor cells may be located more superficially in the epithelium (Coates *et al.*, 1996). In this study, the average baseline activity of SARs was not significantly altered by acetazolamide administration; a slight increase in baseline activity occurred in five SARs but in the remaining three SARs the baseline activity was decreased. Blockade of CA activity is thought to elicit a disequilibrium in the CO_2 transport system, giving rise to increased CO_2 tension and decreased pH level in the tissue. Furthermore, acetazolamide has the effect of decreasing the availability of protons for Na^+–H^+ exchange. Acetazolamide treatment is therefore

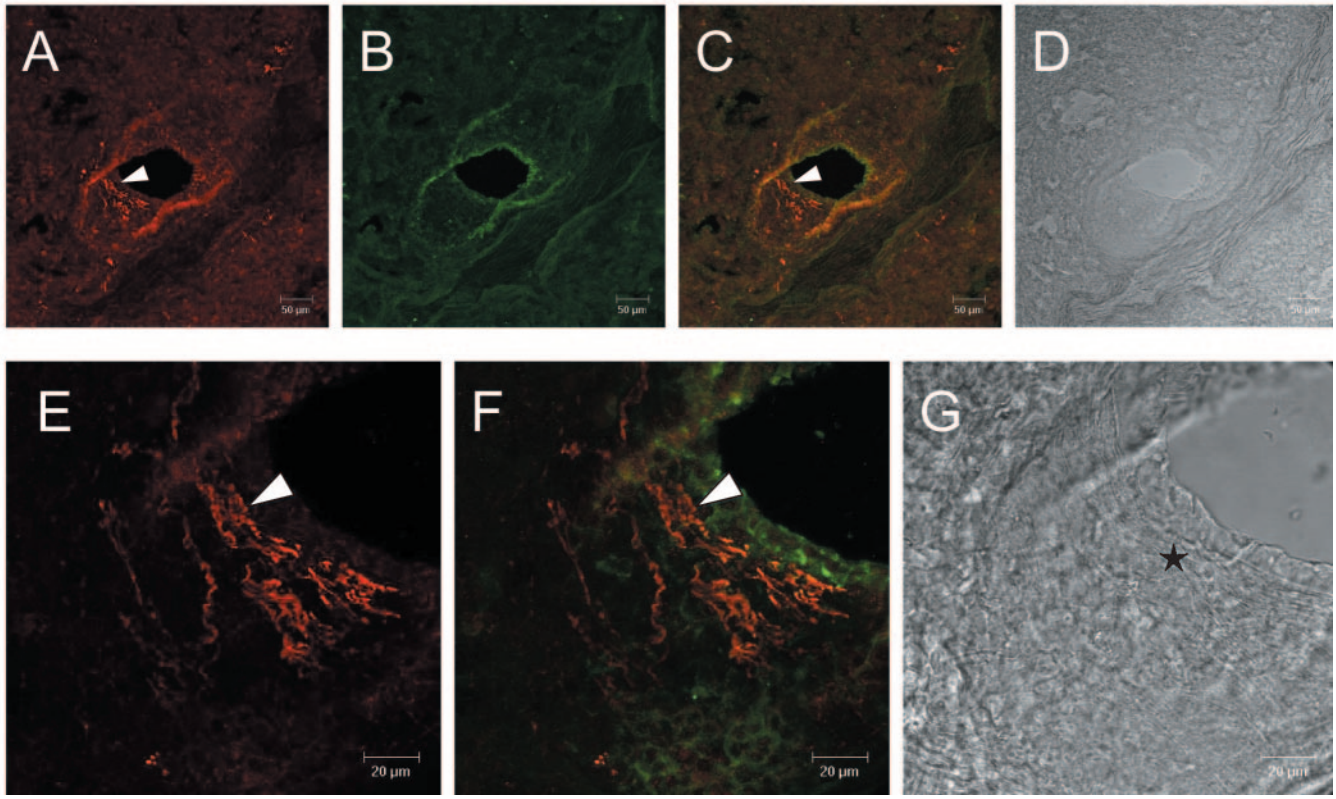

Figure 7 **(A)** Confocal images of the receptor structure (arrow head) identified by PGP 9.5-immunoreactive staining (red) in a brochioles wall. **(B)** CA-isozyme-II-immunoreactive staining (green) in the same section. **(C)** double-immunoreactive stainings of PGP 9.5 and CA isozyme II. **(D)** Serial section (no stainings) of panels A–C. **(E)** A higher magnification of panel A. **(F)** A higher magnification of panel C. **(G)** A higher magnification of panel D. The bronchiole wall included a thin layer of smooth muscles (star). Note that the receptor terminals with positive staining of PGP 9.5 (red) were leaflike but they did not correspond with positive staining of CA isozyme II (green).

considered to decrease pH in the SAR endings as well as in the vicinity of the endings and the decrease in pH may be predominant over 4-AP-like actions and as a result, would inhibit SAR activity. This resultant effect of pH on the SAR activity is probably explained by the fact showing that CO_2-induced SAR inhibition in acetazolamide-treated animals became more pronounced in the latter period of CO_2 inhalation. The results of this study correspond with the observations in the rat, which indicated that the differential effect of acetazolamide on a baseline discharge of SARs (Lai and Kou, 1998). Presumably, acetazolamide effect on SARs may reflect the difference between the magnitudes of decreased I_A in the SAR terminals as well as decreased pH levels in the receptor or in the tissue.

On the other hand, the absence of wood smoke-induced SAR inhibition in the rat may be related to a lack of CA at the receptor sites (Lai and Kou, 1998). In this study, we found that the SAR structures, showing the PGP 9.5 immunoreactivity, have multiple endings to appear leaflike and these receptors were located in the bronchiole at different diameters. Furthermore, some SAR structures are buried in the airway smooth muscle. Others located in a branchial wall, including the smooth muscle layer, had knoblike extensions. These observations were consistent with those reported by Yu *et al.* (2003) in the rabbit. In the same sections, the expression of CA-isozyme-II-like immunoreactive SAR structures was not identified. This does not implies that CO_2-induced SAR inhibition is not due to the CA-dependent CO_2 hydration effect. For this reason, the CA isozyme II is one of the erythrocyte CAs. In addition, there is evidence that the membrane-associated CA, CA isozyme IV, is immunohistochemically identified in the capillary endothelium in the rat lung, indicating that this type in CA isoenzymes plays an important role in preventing pH disequilibrium during tissue as well as alveolar CO_2 exchange (Fleming *et al.*, 1993).

In conclusion, the inhibitory response of SAR activity to CO_2 inhalation was blocked by pretreatment with either 4-AP or acetazolamide and the blocking action of acetazolamide was smaller than that seen after 4-AP treatment. Furthermore, the expression of CA-isozyme-II-like immunoreactive SAR structures, given the potential contribution of a cytosolic form, but not a membrane from, was not found in the bronchiole at different diameters. The results lead us to suggest that CO_2-induced SAR inhibition may be explained by the intracellular pH mechanism in SAR

terminals and/or by the activation of 4-AP sensitive-K^+ currents on those endings. Further studies are needed to determine whether acetazolamide application activates the transient K^+ (I_A) current on SAR terminals, which is sensitive to 4-AP.

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