

The inhibition of sodium influx attenuates airway response to a specific antigen challenge

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1 We have previously observed that manipulation of Na⁺ availability during passive *in vitro* sensitization altered electrophysiological and contractile changes of airway smooth muscle cells. The purpose of this study was to establish whether interference with Na⁺ influx during sensitization also influences the response of airway smooth muscle, both *in vivo* and *in vitro*, to a specific antigen challenge.

2 Isolated segments of trachea which had been sensitized to ovalbumin in the presence of the Na⁺ channel-blocking agent amiloride (10⁻⁵ M) showed no electrical or contractile response to ovalbumin in spite of their ability to respond to histamine (10⁻⁵ M).

3 Airway smooth muscle preparations sensitized to ovalbumin in a Na⁺-deficient medium failed to show any contractile response after exposure to ovalbumin and only a small depolarization of airway smooth muscle cells was detected.

4 Guinea-pigs were passively sensitized *in vivo* either in the absence of, or following pretreatment with, amiloride (1 mg kg⁻¹ s.c.). These animals were then exposed to an ovalbumin inhalation challenge and both lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were measured.

5 After an inhalation challenge of sensitized animals, we observed a significant increase in lung resistance (R_L) achieving a maximum of 489% of the baseline values and a decrease in dynamic lung compliance (C_{dyn}). Twenty min after ovalbumin challenge C_{dyn} was equivalent to 20% of baseline values.

6 In animals pretreated with amiloride during sensitization, the inhalation challenge caused only a small increase in R_L achieving a maximum increase of 148% of baseline values, and a small decrease in C_{dyn} . Twenty min after ovalbumin challenge C_{dyn} was equivalent to 98% of baseline values.

7 We concluded that interference with Na⁺ influx during both *in vitro* and *in vivo* sensitization attenuates the contractile and electrical responses of airway smooth muscle preparations or the changes in lung resistance and compliance observed after antigen challenge.

Introduction

Recently, we have shown that both active and passive sensitization resulted in changes in the membrane potential of airway smooth muscle cells (Souhrada & Souhrada, 1981; 1985). All the evidence suggests that serum from sensitized animals alters airway smooth muscle cell membrane permeability leading to an increased Na⁺ influx into the airway smooth muscle cells. Furthermore, we have established that this Na⁺ influx initiates a series of events leading to changes in electrophysiological properties, subsequent stimulation of Na/K-ATPase, hyperpolarization of the cell membrane and an increased

contractile response of airway smooth muscle cells to agents such as histamine (Souhrada & Souhrada, 1981; 1984). The alteration of airway smooth muscle cell membrane properties induced by serum in sensitized animals occurred in a dose-dependent manner; membrane changes were still observed at a dilution of sensitized serum equal to 1:10 000 (Souhrada & Souhrada, 1987a). Recent experiments confirmed that similar changes in membrane properties can also be observed if, during passive *in vitro* sensitization, purified guinea-pig IgG₁ antibodies are used instead of sensitized serum (Souhrada & Souhrada, 1987b).

In view of these data, we speculated whether interference with this sensitization-induced Na⁺ influx

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might also influence the response of immunized airway smooth muscle cells to a specific antigen challenge. We therefore tested the effect of the Na^+ -channel blocker amiloride, as well as the effect of a Na^+ -deficient medium, on the changes in the resting membrane potential and functional status of airway smooth muscle cells observed during sensitization. Also, we investigated whether these procedures can influence both the electrical and contractile responses of airway smooth muscle cells observed after subsequent antigen challenge. Furthermore, we examined whether treatment of animals with amiloride during sensitization, could alter the *in vivo* response of sensitized animals to the specific antigen inhalation challenge.

Methods

Preparation of sensitized serum

Camm-Hartley male guinea-pigs, body weight 350–450 g, were used in all experiments. They were sensitized as previously described (Souhrada & Souhrada, 1981; 1985). Briefly, the animals received 1.0 ml of *pertussis* vaccine i.p. and 1.0 ml of 1% albumin (grade V₁, salt-free crystallized and lyophilized – Sigma) administered subcutaneously; 14 days were allowed for the immunization (acute sensitization). After this period, the animals were reinjected subcutaneously with 0.25 ml of 0.1% albumin and this injection was repeated twice for two consecutive weeks. One week after the second sensitization the animals were bled and the sensitized serum stored at 4°C.

Simultaneous measurement of the resting membrane potential and isometric force of airway smooth muscle cell preparations

Details of this technique have been described elsewhere (Souhrada & Souhrada, 1981; 1985). Briefly, after killing the animals by cervical dislocation, mid-cervical portions of each trachea were rapidly dissected and placed in oxygenated physiological salt solution at 37°C. Subsequently, these tracheal segments were cut open along the ventral surface and attached through the cartilaginous rings to the base of the experimental chamber using a metal clamp. A second mechanical clamp was attached to an L-shaped holder which could be moved in a horizontal direction with a micrometre. A pair of strain gauges was placed on this holder so that the development of any isometric force between clamps could be readily recorded. Preparations were allowed to equilibrate in the physiological salt solution for 45–60 min before repeated measurements of the resting mem-

brane potential (one impalement at each time point) and continuous monitoring of the isometric force were initiated. Individual values of resting membrane potential as obtained at each time point from different tissues were used to derive the mean \pm s.e.

Using a Gilson peristaltic pump, the experimental chamber (Boyd & Martin, 1956) was constantly perfused (5 ml min⁻¹) with a physiological salt solution of the following composition (in mM): NaCl 117.5, KCl 5.37, CaCl₂ 2.52, MgSO₄ · 7H₂O 0.56, NaH₂PO₄ 1.17, NaHCO₃ 15.51, glucose 5.50 and sucrose 13.65, at 37 \pm 0.5°C. The solution in the chamber was equilibrated with a gas mixture of 95% O₂ and 5% CO₂, so that the P_{O₂}, and P_{CO₂}, and pH of the physiological salt solution were 550 \pm 5 Torr, 38 \pm 2 Torr, and 7.36 \pm 0.02, respectively.

Single trachealis muscle cells were impaled using glass microelectrodes (W-P Instruments, borosilicate glass LB120F-4) filled with 3M KCl and with a tip potential of less than 10 mV. Only electrodes with a resistance of 80–90 M Ω were used. Electrical signals were conducted via silver/silver chloride electrodes to the input probe of a high-impedance amplifier (W-P Instruments model M4A). The amplifier output was displayed on a storage oscilloscope (Tektronix, model 5103N), and permanent records were obtained either by photographing the oscilloscope trace (C-5 oscilloscope camera, Tektronix), or by simultaneously recording the amplifier output on a pen recorder (Beckman R611). The reference electrode was a silver/silver chloride electrode placed in the bottom of the experimental chamber. Details of this technique and of the experimental set-up have been described previously (Souhrada & Souhrada, 1981). Successful impalement of the cell was indicated by: (1) prompt negative deflection in the electrical record; (2) maintenance of steady potential for 10 s or more; and (3) immediate return to baseline upon withdrawal of the electrode from the cell (Taylor *et al.*, 1969). At the beginning of the experiment the resting tone of all preparations was adjusted to 2.0 \pm 0.1 g.

Passive in vitro sensitization of guinea-pig trachea

Passive *in vitro* sensitization of airway smooth muscle preparations was performed by an Austen technique (Austen, 1973). In brief, tracheal segments isolated from normal, non-sensitized animals were incubated with 1:10 dilution of serum, obtained from repeatedly sensitized animals (see above) for two hours at 37°C, followed by repeated washout (5 min) of the experimental chamber with a physiological salt solution. Then the resting tone and resting membrane potential were determined, and 0.1% purified ovalbumin was administered into the

experimental chamber containing the sensitized airway smooth muscle preparation. After the administration of antigen, both electrical and contractile changes were recorded.

Effect of amiloride

After a 60 min incubation period the resting tone of tracheal preparations was adjusted to 2.0 ± 0.2 g and then resting membrane potential was ascertained. Determination of these two variables was repeated 30 min later. In most experiments amiloride (10^{-5} M) and sensitized serum (1 : 10 dilution) were introduced into the experimental chamber simultaneously and measurements of resting membrane potential and isometric force were repeated after 15–20 min. Administration of amiloride and sensitized serum caused no significant changes at this time in either of these two variables. A concentration of 10^{-5} M amiloride was selected as previous *in vitro* data suggested that, at this concentration, amiloride exhibits near maximal inhibition of Na^+ transport across cell membranes (Bentley, 1968; Benos, 1982). Two hours after introduction of amiloride and sensitized serum, tracheal segments were exposed to 0.1% of ovalbumin and 15 min later, following washout of the experimental chamber with a fresh physiological salt solution, to a single concentration of 10^{-5} M histamine. At predetermined time intervals, both isometric force and resting membrane potential were determined.

In a separate series of experiments a similar protocol was followed except that amiloride was added to the experimental chamber at various times after the start of tissue exposure to sensitized serum.

Effect of Na^+ replacement

The effect of Na^+ replacement on the response of sensitized tracheal segment to ovalbumin and histamine followed a time sequence similar to that described for amiloride. After determination of the isometric force and the resting membrane potential, a physiological salt solution containing a normal quantity of Na^+ was replaced by physiological salt solution in which sodium chloride was substituted with an equimolar amount of choline chloride (118 mM). Simultaneously with the low Na^+ physiological salt solution, sensitized serum (1 : 10 dilution) was introduced into the experimental chamber. After a two hour sensitization period, tissue was challenged with a 0.1% ovalbumin and 15 min later, following a washout, exposed to a single concentration of 10^{-5} M histamine. At predetermined time intervals, both isometric force and resting membrane potential were determined.

In vivo assessment of pulmonary function

Dynamic lung compliance (C_{dyn}) and pulmonary resistance (R_L) were determined in a constant volume body plethysmograph by use of methodology previously described (Hulbert *et al.*, 1985).

The animals were anaesthetized with sodium pentobarbitone (Nembutal; 35 mg kg^{-1} , administered intraperitoneally) and placed inside the body plethysmograph in a supine position. Transpulmonary pressure was determined as the difference between oesophageal and tracheal pressures, by a differential pressure transducer (Statham P23V) (Boyd & Mangos, 1981; Hulbert *et al.*, 1985). One port of this transducer was connected to the oesophageal catheter, while the other was connected to a side arm of the tracheal cannula. The oesophageal catheter was constructed from PE No. 190 polyethylene tubing and was positioned in the oesophagus so that maximal negative swings in pressure were observed. The position of the catheter was repeatedly checked through the C_{dyn} and R_L measurement. Movement of air in and out of the body plethysmograph was detected with a Fleish No. 1 pneumotachograph attached to the wall of the body plethysmograph. The pneumotachograph was coupled to a differential pressure transducer (Statham P97), with the output displayed on a Beckman multichannel recorder. The same signal was also electronically treated using a Beckman No. 9873B integrator to display tidal volume. At the beginning and end of each experiment flow, volume and pressure channels were calibrated. The values of dynamic compliance (C_{dyn}) and respiratory resistance (R_L) were calculated as previously described (Von Neergaard & Wirz, 1927). During the inhalation challenge the tracheal cannula was connected to a small plexiglass box ($13 \times 20 \times 30$ cm) into which an aerosol of 0.1% ovalbumin was delivered for a period of 45 s. To administer ovalbumin in aerosol form we used a DeVilbiss nebulizer operated by compressed air using an air flow of 20 cc min^{-1} .

Passive in vivo sensitization

Normal (Camm-Hartley) male albino guinea-pigs (BW 400–500 g) were passively sensitized *in vivo* by use of a modified protocol of Mongar & Schild (1960). The control group of animals received an intraperitoneal injection (2 ml) of sensitized serum (see above) over two consecutive days. Then, after 24 h, these animals were exposed to the inhalation challenge of a specific antigen (0.1% purified ovalbumin, Sigma). The experimental group was sensitized in a similar manner. However, one hour before each sensitization, these animals received a subcutaneous

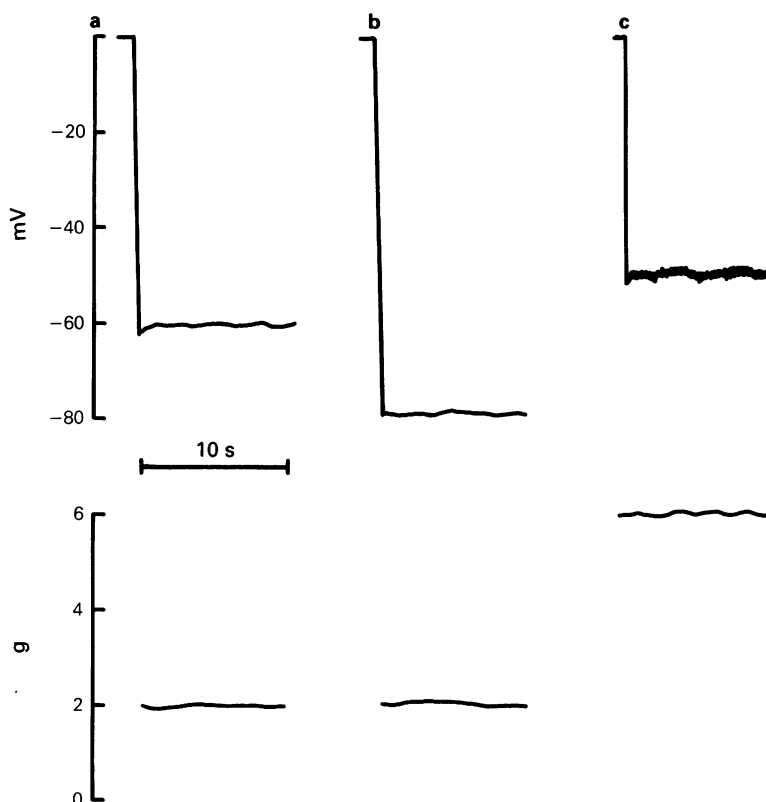


Figure 1 The electrical and mechanical activity of airway smooth muscle (m. trachealis transversus) observed after sensitization and after specific antigen challenge. Electrical recordings were obtained from three different airway smooth muscle cells (upper trace) while the lower trace shows the isometric force developed by a segment of trachea at the time of impalement. Activities were recorded (a) before, (b) 2 h after the addition of 1:10 diluted sensitized serum, and (c) 10 min after addition of ovalbumin (0.1%) into the experimental chamber.

injection of amiloride (1 mg kg^{-1}). This dose of amiloride was based on a previously established *in vivo* effective diuretic dose of amiloride (Compos *et al.*, 1966). As in the control group, 24 h after the last sensitizing injection, animals in the experimental group were exposed to the inhalation challenge with aerosol of 0.1% ovalbumin. Before and after the inhalation challenge, pulmonary function was monitored for a period of 20 min.

Evaluation of data

Data are expressed as mean \pm s.e. Statistical analysis of changes in both the resting membrane potential and the isometric force included 6–8 separate tracheal preparations analysed at preselected time points. A difference between groups was examined by use of analysis of variance with repeated measures. Statistical significance was established at a 5% level.

Results

Figure 1 shows a change in the resting membrane potential of three different airway smooth muscle cells and corresponding values of isometric force in a typical tracheal preparation ascertained at baseline – in normal conditions; after sensitization (with 1:10 dilution of sensitized serum) and after 0.1% ovalbumin challenge.

Figure 2 illustrates the effect of an ovalbumin challenge (0.1% ovalbumin) on the resting membrane potential and the isometric force of six sensitized airway smooth muscle preparations. As previously demonstrated, a passive *in vitro* sensitization caused a significant ($P < 0.001$) increase in the resting membrane potential from $-60.6 \pm 0.6 \text{ mV}$ to $-68.6 \pm 0.7 \text{ mV}$. After sensitization the resting tone of tracheal preparations was similar to the presensitization level of tone. The addition of 0.1% ovalbumin

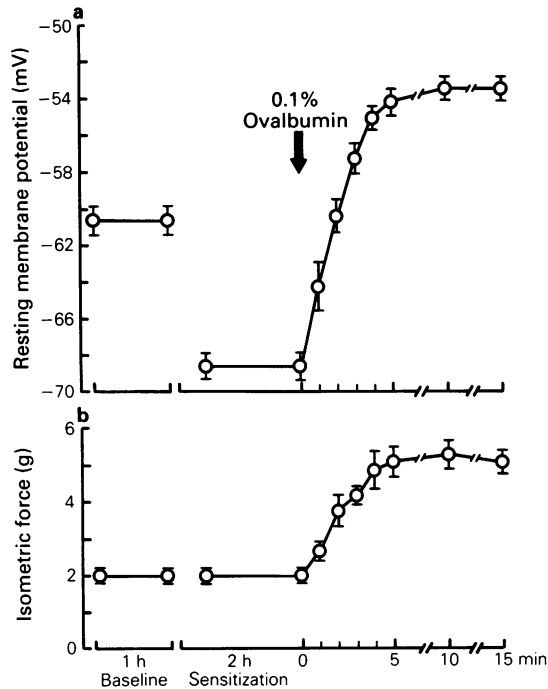


Figure 2 The effect of passive *in vitro* sensitization (1:10 dilution of immunized serum) and specific antigen challenge (0.1% ovalbumin) on (a) electrical and (b) mechanical responses of segments of guinea-pig trachea. Data represent means of values from six different tracheal preparations; vertical lines indicate s.e.

into the experimental chamber caused an immediate, significant ($P < 0.001$) depolarization of airway smooth muscle cells. The membrane potential achieved a steady state of -53.3 ± 0.4 mV at 10 min. During this time, the isometric force increased to a peak value of 5.5 ± 0.5 g ($P < 0.05$).

Figure 3 illustrates the effect of amiloride (10^{-5} M) on the passive *in vitro* sensitization and the consecutive electrical and mechanical responses of sensitized tissues to 0.1% ovalbumin. With amiloride pretreatment, a passive *in vitro* sensitization failed to affect significantly the resting membrane potential of airway smooth muscle cells. Furthermore, after the addition of 0.1% ovalbumin, only a small non-significant depolarization of airway smooth muscle cells was detected, with essentially no change in the isometric force of the same preparations. After the isometric force and membrane potential had achieved a steady state we also examined the response of these preparations to a single concentration of histamine (10^{-5} M). After histamine, we observed a significant ($P < 0.01$) depolarization and an increase ($P < 0.001$) in the isometric force.

Since amiloride treatment may have some non-

specific effects on airway smooth muscle cells, we also conducted passive *in vitro* sensitization when the availability of Na^+ in the physiological salt solution was substantially decreased. In these experiments, sodium chloride of the physiological salt solution was replaced with choline chloride (118 mM). As in the previous experiment, replacement of sodium chloride with choline chloride prevented the changes in the resting membrane potential induced by passive *in vitro* sensitization (see Figure 4). Furthermore, consecutive administration of ovalbumin failed to excite these airway smooth muscle preparations. However, as can be seen in Figure 4, the same preparations showed a significant depolarization ($P < 0.001$) and an increase in the isometric force after histamine (10^{-5} M) administration ($P < 0.01$).

Figure 5 summarizes the effect of a specific antigen inhalation challenge on the changes of dynamic lung compliance (C_{dyn}) of animals which had been passively sensitized with and without amiloride pretreatment. Before antigen challenge C_{dyn} was equal to 0.25 ± 0.05 ml $\text{cmH}_2\text{O}^{-1}$. Animals not pretreated with amiloride showed a rapid decrease ($P < 0.001$) in C_{dyn} , reaching a steady state value 10 to 12 min after the end of the inhalation challenge. Twenty min after inhalation challenge in this group C_{dyn} was equivalent to $20 \pm 12\%$ of the baseline values, suggesting the presence of severe obstruction in small airways. The animals pretreated with amiloride, however, showed a non-significant decrease in C_{dyn} after the inhalation challenge. Twenty min after inhalation challenge C_{dyn} in this group was equivalent to $98 \pm 2\%$ of the baseline values.

Figure 6 illustrates the parallel change in the lung resistance (R_L). Before antigen challenge R_L was equal to 0.55 ± 0.06 cm $\text{H}_2\text{O ml}^{-1} \text{ s}^{-1}$. In a group of passively sensitized animals, 10 min after the end of the inhalation antigen challenge, a significant ($P < 0.01$) increase in R_L to $489 \pm 85\%$ of the baseline was observed. Twenty min after the inhalation challenge, the value of R_L was equivalent to $350 \pm 60\%$ of the baseline. In contrast, in a group of sensitized animals pretreated with amiloride, 4 min after antigen inhalation challenge R_L transiently increased to only $148 \pm 5\%$ of the baseline. Twenty min after the inhalation challenge the value of R_L was essentially the same as that observed before challenge. Furthermore, amiloride-pretreated, sensitized animals showed no symptoms of any respiratory distress.

Discussion

In this study we have demonstrated that pretreatment of airway smooth muscle preparations of

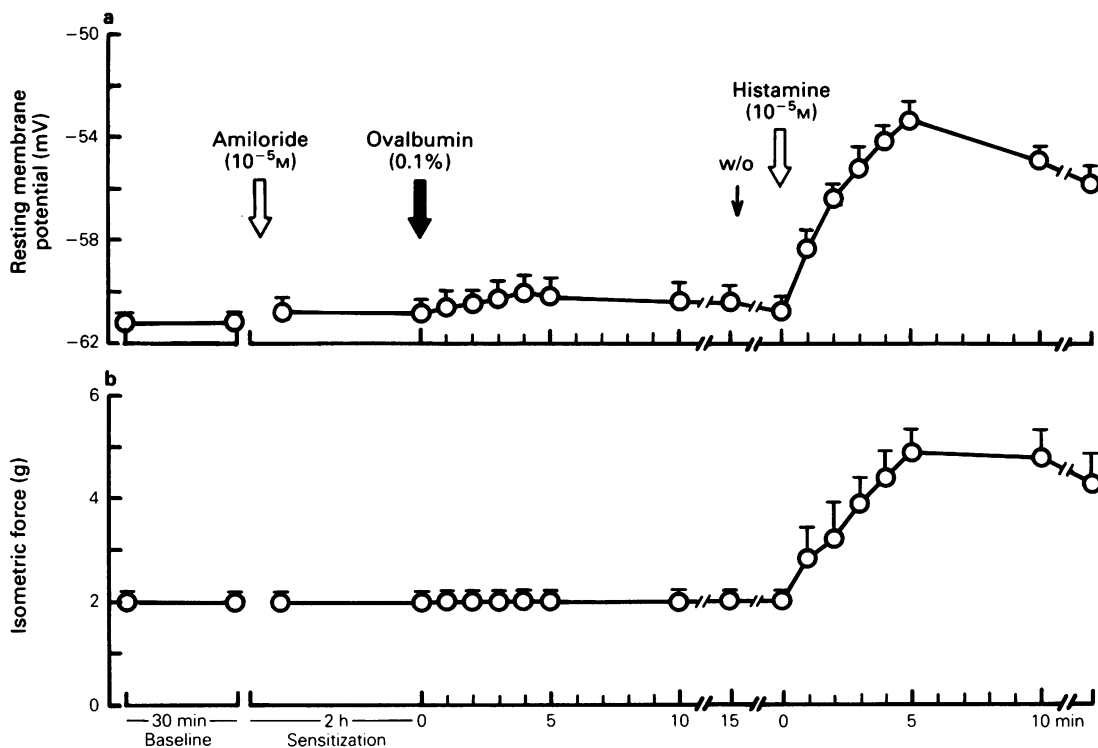


Figure 3 Effect of specific antigen challenge (0.1% ovalbumin) and histamine (10^{-5} M) administration on (a) electrical and (b) mechanical responses of segments of guinea-pig trachea pretreated with amiloride (10^{-5} M). Data represent means of values from six different tracheal preparations; vertical lines indicate s.e.

guinea-pigs with amiloride before sensitization, or decreasing the availability of Na^+ in the experimental medium, prevented or substantially attenuated contractile and electrical responses of these cells to a specific antigen challenge. The response to antigen challenge is known as a Schultz-Dale response (Schultz, 1910; Dale, 1913).

In the absence of any direct evidence available in this study which suggests that amiloride inhibits Na^+ influx, it can be argued that the effect of amiloride on the airway smooth muscle may be non-specific. However, this seems unlikely since a similar effect on the antigen-induced response was also seen in experiments in which a Na^+ -deficient medium was used. These experiments and extensive evidence in the literature (for review see Benos, 1982) suggest that the effect of amiloride is related to specific inhibition of Na^+ influx into the airway smooth muscle cells.

It can be concluded that the observed attenuation of the antigen response is related to inhibition of amiloride-sensitive Na^+ channels. Inhibition of Na^+

influx could interfere with at least three different events: (a) excitation-contraction mechanism of airway smooth muscle cells; (b) the process of sensitization, i.e., the attachment of specific reaginic antibodies on specific cells (including airway smooth muscle cells) with F_c receptors; and possibly also, (c) the process of mediator release from mast and other cells.

It is unlikely that amiloride pretreatment interfered with the excitation-contraction mechanism and thus directly inhibited the response of airway smooth muscle to ovalbumin. We have shown that all amiloride pretreated preparations responded to a single concentration of histamine, which suggests that the excitation-concentration mechanism of airway smooth muscle cells was not damaged.

One can speculate that amiloride may have interfered with the process of sensitization, specifically with the binding of reaginic antibodies on F_c receptors of cells such as mast cells. It can be postulated that the occupancy of the sodium channel could have prevented the attachment of reaginic antibodies

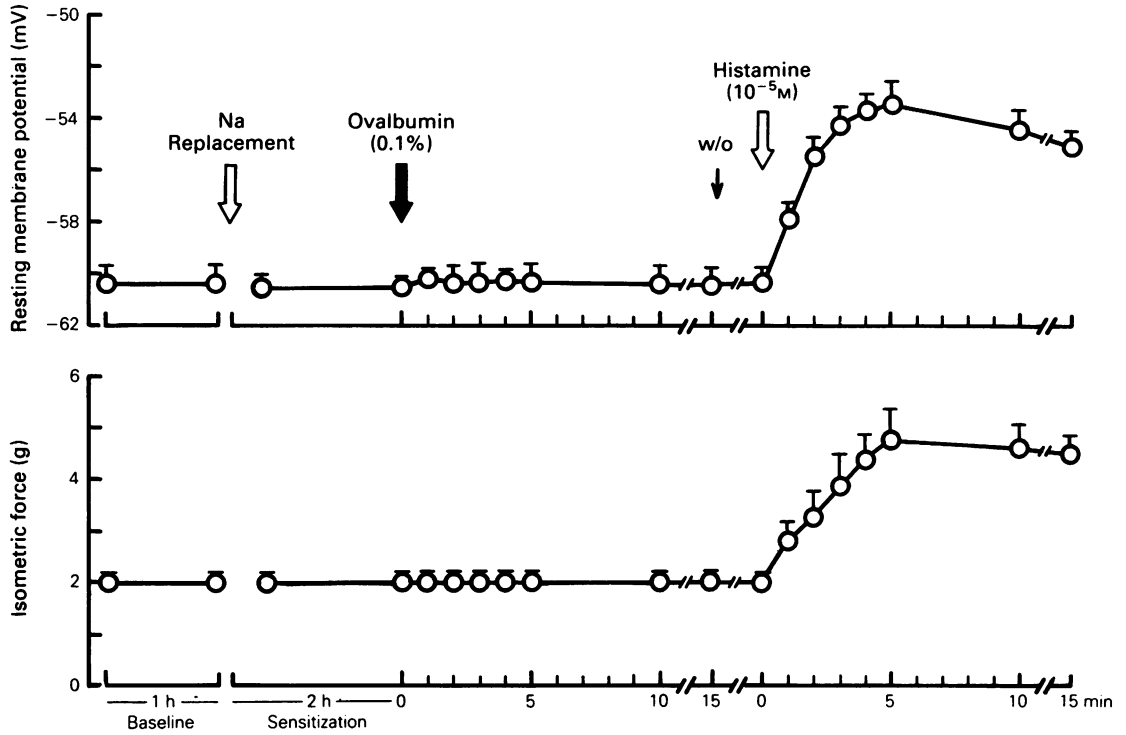


Figure 4 Effect of specific antigen challenge (0.1% ovalbumin) and histamine (10^{-5} M) administration on (a) electrical and (b) contractile responses of segments of guinea-pig trachea sensitized in a Na^+ -deficient environment (sodium chloride in physiological salt solution was replaced with choline chloride). Data represent means of values from eight different tracheal preparations; vertical lines indicate s.e.

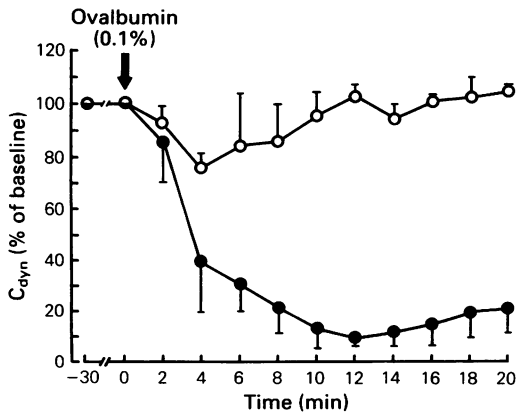


Figure 5 Effect of 0.1% ovalbumin aerosol (45 s) on dynamic lung compliance (C_{dyn}), expressed as % of baseline in sensitized (\bullet) and sensitized plus amiloride-treated (\circ) guinea-pigs. Before antigen challenge C_{dyn} was equal to $0.25 \pm 0.05 \text{ ml cmH}_2\text{O}^{-1}$. Data represent means of values from six animals; vertical lines indicate s.e.

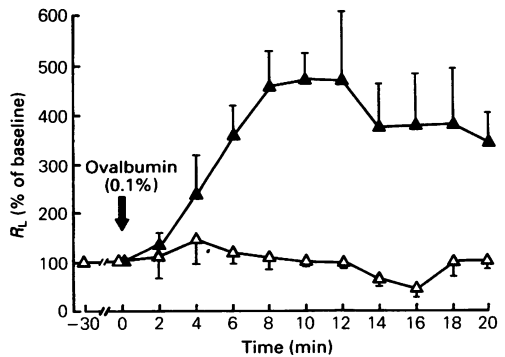


Figure 6 Effect of 0.1% ovalbumin aerosol (45 s) on lung resistance (R_L) expressed as % of baseline in sensitized (\blacktriangle) and sensitized plus amiloride-treated (\triangle) guinea-pigs. Before antigen challenge R_L was equal to $0.55 \pm 0.06 \text{ cmH}_2\text{O ml}^{-1} \text{ s}^{-1}$. Data represent means of values from six animals; vertical lines indicate s.e.

to F_c receptors and therefore tissue sensitization would be prevented. Indeed, it has been demonstrated that occupancy of F_c receptors with specific reaginic antibodies can lead to the opening of the sodium channel (Young *et al.*, 1983), a finding which suggests a relationship between F_c receptors and the sodium channel. Data obtained in the present study show that amiloride or Na^+ -deficient medium prevented both the electrical and contractile changes of airway smooth muscle cells observed during sensitization. These findings thus suggest that the availability of Na^+ and the normal function of the Na^+ -channel are critical for the *in vitro* sensitization of airway smooth muscle cells.

Preliminary experiments showed that the inhibition of contractile and electrical responses to the ovalbumin was dependent on the time when amiloride was administered into the experimental chamber. In contrast to the results obtained when amiloride was administered before sensitization (i.e. no response to ovalbumin was detected), the administration of amiloride during or after sensitization failed to influence the response of airway smooth muscle to ovalbumin. In view of this it may be concluded that amiloride administration interfered with the process of sensitization.

The present study did not address the issue of changes of the resting membrane potential and the isometric tone described during sensitization. As demonstrated previously (Souhrada & Souhrada, 1985; 1987a), a decrease in sodium availability during sensitization has an inhibitory effect on the sensitization-induced changes in the membrane potential (both, transient depolarization and steady-state hyperpolarization) and the isometric force.

Alternatively, amiloride administration could interfere with the release of mediators of anaphylaxis, such as the release of histamine from the mast cells. Indeed, in the guinea-pig, histamine is a major mediator of anaphylaxis. If histamine and other mediators were released after the ovalbumin inhalation challenge, one would expect these airway preparations to develop tension and depolarize. It has been shown that histamine causes airway smooth muscle cells to depolarize and contract (Souhrada & Souhrada, 1985). However, in the presence of amilo-

ride we did not observe any significant change in the airway smooth muscle tone, which suggests that no histamine or other mediators of anaphylaxis were released after ovalbumin challenge. Therefore, it can be postulated that amiloride interferes with the release of mediators of anaphylaxis from specific cells including mast cells. These are undoubtedly present in all tracheal preparations (Souhrada & Souhrada, 1983).

As previously demonstrated (McCaig & Souhrada, 1980) normal guinea-pig airway smooth muscle exhibits three different types of electrical behaviour; 27% of cells show regular spontaneous electrical activity; 44% of cells exhibit irregular fluctuation of membrane potential and the remaining cells show no electrical activity. In the present study, a resting membrane potential was only analysed in cells which showed no electrical activity. However, we have observed that after *in vitro* sensitization there was a general decrease in the incidence of regular spontaneous electrical activities while the incidence of irregular activities usually increased. This observation is similar to that observed after *in vivo* sensitization of guinea-pigs with specific antigen (Souhrada & Souhrada, 1981).

Amiloride HCl is a potassium-sparing diuretic that inhibits sodium transport across amphibian skin, renal distal tubular cells, epithelial cells of the colon, erythrocytes and many other cells (Benos, 1982). This action of amiloride is very specific for the sodium channel (Compos *et al.*, 1966). The action is rapid in onset and the binding of amiloride to the sodium channel is reversible (Cuthbert & Shum, 1974).

In humans, amiloride is used as a diuretic in the treatment of hypertension and congestive cardiac failure. One can only speculate whether this agent, in addition to the increased excretion of sodium, chloride and water, may also have some use in attenuating the bronchoconstrictor response to a specific antigen in patients with allergic asthma.

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