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The obligatory role of calcium in the development of antigen-induced airway hyperreactivity to cold provocation in the rat isolated trachea

[1]N. Chand, W. Diamantis & R.D. Sofia

Department of Pharmacology, Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, New Jersey 08512 U.S.A.

1 The effect of antigen challenge on cold provocation (30–10°C) in isolated tracheal segments from control (normal) and ovalbumin-sensitized rats was studied.

2 Sensitization alone or acute cold provocation (10°C) alone was not sufficient to cause contraction of tracheal smooth muscle preparation.

3 Following recovery from antigen-induced responses (Schultz-Dale phenomenon) in sensitized rat segments, cold provocation induced strong contractile responses.

4 Both the antigen-induced contractions and the subsequent development of airway hyperreactivity to cold were dependent on extracellular Ca^{2+} and inhibited by verapamil.

5 The data in this study indicate that extracellular Ca^{2+} plays an obligatory role in the mediation of antigen-induced contractile responses as well as the subsequent development of hyperreactivity to cold provocation in rat tracheal smooth muscle.

Introduction

The precise mechanism of exercise-induced asthma (EIA) is not yet known. One hypothesis is that respiratory heat loss and subsequent cooling of the airways constitute the initial reaction sequence causing bronchoconstriction in asthmatics (Anderson, 1984; Bar-Yishay & Godfrey, 1984; McFadden & Ingram, 1984).

Calcium is known to play an essential role in a multitude of biological processes, e.g. excitation-contraction coupling in smooth muscles, stimulus-secretion coupling in leukocytes/mast cells/basophils, mucus production, etc. Regardless of the triggering stimuli, e.g. allergens, bacterial-viral infections, exercise and chemical mediators, the characteristic airway hyperreactivity observed in allergic respiratory diseases will ultimately be a result of an increased level of intracellular Ca^{2+} interacting with the contractile machinery (Weiss & Viswanath, 1979; Andersson, 1983; Triggle, 1983; Chand *et al.*, 1986b). Calcium channel blockers have been found to exert beneficial effects in EIA (Patel, 1981; Cerrina *et al.*, 1981; Corris *et al.*, 1983). In earlier studies, cooling of isolated bronchial or tracheal smooth muscle preparations of man, dog, guinea-pig and rat has been demonstrated to potentiate contractile responses to histamine, acetylcholine (ACh) or methacholine, etc. (Souhrada & Souhrada, 1981; Murlas *et al.*, 1982; Tran *et al.*, 1982; Ishii & Shimo, 1985). In the present study, the effect of antigen challenge on cold-induced responses in rat isolated tracheal segments was investigated. Furthermore, the role of Ca^{2+} in the mediation of antigen-induced responses (Schultz-Dale phenomenon) (Chand & Eyre, 1978) and subsequent development of airway hyperreactivity to cold provocation in rat isolated tracheal segments (Chand *et al.*, 1986a) was also studied.

Methods

Adult male Sprague-Dawley rats weighing 350–550 g were used in this study. Most of these rats were sensitized with an intraperitoneal injection of 1 mg ovalbumin (OA) and 2.2×10^9 killed *Bordetella pertussis* organisms (Mota, 1964). Fourteen to 21 days later, OA-sensitized as well as normal 'unsensitized' rats were killed by CO_2 exposure. Approximately 8 to 10 mm long terminal segments of the trachea were

[1] Author for correspondence.

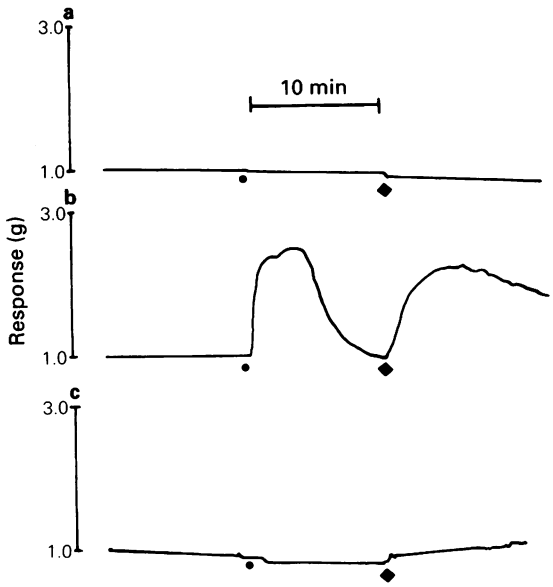

Figure 1 Typical tracings showing (a) non-responsiveness of normal (unsensitized) rat tracheal segment to antigen (ovalbumin 1 $\mu g\,ml^{-1}$ added at ●) and subsequent cold (10°C) provocation (◆); (b) ovalbumin-sensitized tracheal segment reacted with contractile response to antigen challenge (●) and subsequently exhibited airway hyperreactivity to cold (◆); (c) a sensitized tracheal segment which failed to respond to antigen challenge (● non-responder) subsequently reacted with a weak contractile response to cold provocation (◆). The maximum responses to acetylcholine (10^{-3} M) were: (a) 4.0 (b) 4.1 and (c) 3.8 g, respectively.

dissected and kept in warm Krebs-Henseleit solution (37°C). Each tracheal tube was cut into two equal segments (3–4 mm), set up 'in pairs' in isolated tissue baths containing Krebs-Henseleit solution, bubbled with a mixture of 95% O_2 and 5% CO_2, and maintained at 37°C by circulating heated water in the outer jacket of isolated tissue baths. (In this study, tissues from ovalbumin-sensitized rats will be referred to as sensitized tissues). The composition of Krebs-Henseleit solution was: (mM) NaCl 119, KCl 4.7, $CaCl_2$. $2H_2O$ 2.5, $MgSO_4$. $7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 10.0 (pH 7.4). Tracheal segments were attached to force-displacement transducers (Grass FT.03C) under 1 g tension. Tissues were allowed to equilibrate for 1 to 3 h with washings at 30–60 min intervals and resting tension was readjusted and maintained during the stabilization period.

Following the equilibration period, sensitized tissues were challenged with OA (1 $\mu g\,ml^{-1}$) producing 42 ± 7% of the ACh-induced maximum response in the data given in Figure 2, and 27 ± 3% of the ACh maximum response in the data given in Figure 4. Generally, antigen-induced contractile responses recovered to baseline levels within 10–20 min after challenge. Following recovery of the allergic responses, circulating water in the outer jacket of the isolated tissue bath was switched to cold water supply (30°, 20° or 10°C; refrigerated constant temperature circulator, Polyscience Series 9000). The cold-induced contractile responses were recorded isometrically for a period of 15 to 30 min or until a plateau was established. The circulation of heated (37°C) water in the outer jacket of tissue baths produced immediate relaxation of cold-induced contractions. Fifteen min later, resting tension (if lowered beyond resting level) was readjusted and then tissues were exposed to ACh (10^{-3} M) to determine the maximum response of each preparation in mg; this response is referred to in the text and graphs as ACh max. The antigen- and cold-induced responses are expressed as % of ACh max.

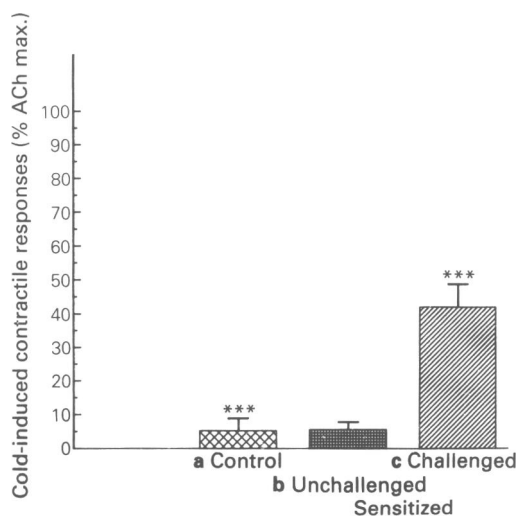

Figure 2 Failure of control (a, non-sensitized, challenged with ovalbumin 1 $\mu g\,ml^{-1}$) and sensitized (b, unchallenged) rat isolated trachea segments to react to cold provocation (10°C). In contrast, immediately after recovery from allergic contractile responses (c, ovalbumin 1 $\mu g\,ml^{-1}$ over a period of 10–20 min) the tracheal segments exhibited remarkable airway hyperreactivity to cold provocation. Each column represents the mean of 8–11 tissues and vertical lines show s.e.mean. ***Indicates significant difference, $P < 0.001$, determined by paired (b vs c) and unpaired (a vs c) t test. Actual responses in mg: (a) 231 ± 155 ($n = 8$), (b) 156 ± 49 ($n = 11$) and (c) 1250 ± 209 ($n = 8$).

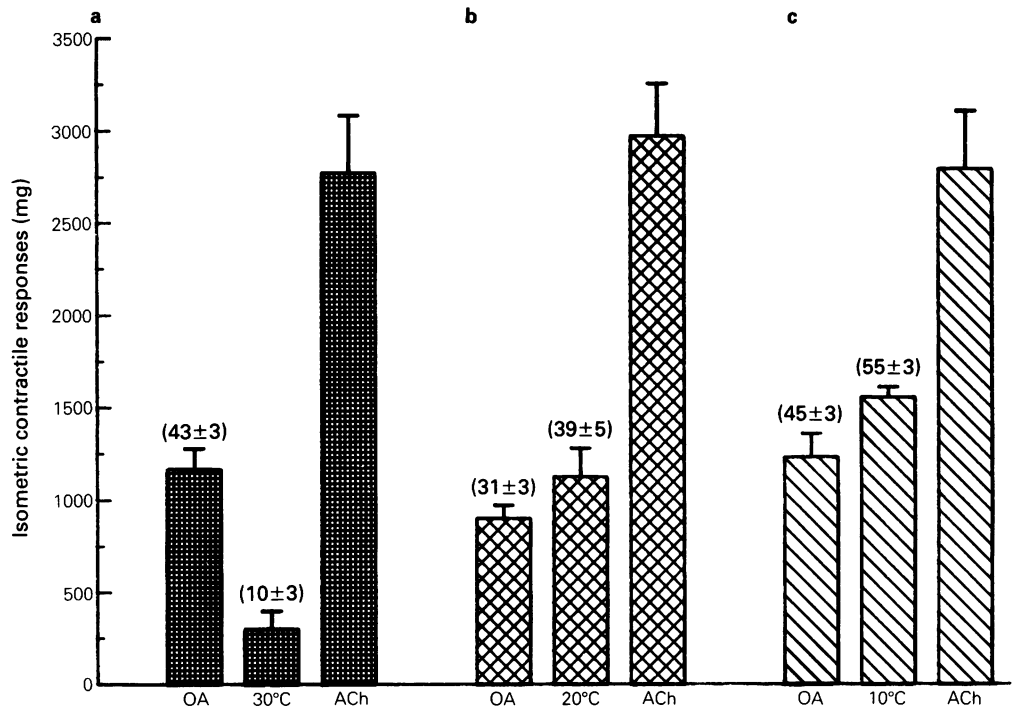

Figure 3 Temperature-dependent contractile responses to cold provocation in isolated tracheal segments obtained from actively sensitized rats, immediately after recovery from allergic responses (OA: ovalbumin 1 $\mu g\,ml^{-1}$ at 37°C). Each segment was exposed to antigen and cold only once. Contractions produced by acetylcholine (ACh, 10^{-3} M) at 37°C are also shown. Each column represents the mean of 6 observations and vertical lines show s.e.mean. Numbers in parentheses above the columns indicate the response as % ACh max.

Role of Ca^{2+} and effect of verapamil on antigen-induced responses and subsequent development of airway hyperreactivity to cold

After the equilibration period in Ca^{2+}-containing Krebs-Henseleit solution, one tracheal segment of each pair was incubated in Ca^{2+}-free Krebs-Henseleit solution for a period of 30 min with two to three washes. The second tissue was maintained in normal (Ca^{2+}-containing) Krebs-Henseleit solution. Both tissues were challenged with OA (1 $\mu g\,ml^{-1}$) and upon recovery from the allergic responses were exposed to cold (10°C) by changing the circulating water in the outer jacket of tissue baths. Fifteen to 30 min later, tissues were exposed to ACh (10^{-3} M) at 37°C in normal Krebs-Henseleit solution.

In other experiments, after the equilibration period, one segment of each pair was exposed to the appropriate concentration of verapamil for a period of 30 min before antigen challenge at 37°C and subsequent cold provocation (10°C) upon recovery from allergic responses. Fifteen to 30 min later tissues were exposed to ACh (10^{-3} M) at 37°C to determine the contractility in mg. If the control segment of a pair exhibited an allergic response less than 10% of ACh max. and/or exhibited poor contractility to ACh (10^{-3} M), i.e., when ACh max. was <1000 mg, then data from such pairs were not included in this study.

The results presented are the means ± s.e.mean and are are expressed as % ACh max. and mg tension. Statistical comparison between control and treated tracheal segments was performed by use of Student's *t* test for paired and unpaired observations; statistical significance was assumed if $P<0.05$.

Asthma is characterized by remarkable airway hyperreactivity to histamine, methacholine, prostaglandin $F_{2\alpha}$, etc. In view of the antigen-induced airway hyperreactivity to cold, it was considered important to examine whether sensitized rat tracheal segments following recovery from allergic responses

also exhibit hyperreactivity to bronchoconstrictor stimuli such as acetylcholine, 5-hydroxytryptamine (5-HT) and KCl. In these experiments the cumulative concentration-effect curves to acetylcholine, 5-HT and KCl were studied in both challenged (following recovery from allergic responses) and unchallenged tracheal segments. The ED_{50} values (i.e., the concentration of bronchoconstrictor agent producing 50% of maximum contractile responses to each agonist) were calculated from the linear part of the concentration-effect curves and compared by the Bliss method (Bliss, 1945).

The following drugs were used in this study: histamine dihydrochloride, ovalbumin (Sigma Chemical Co., St. Louis, MO), acetylcholine chloride (ICN Nutritional Biochemicals, Cleveland, OH), verapamil (Knoll Pharmaceutical Co., Whippany, NJ), 5-hydroxytryptamine creatinine sulphate (5-HT) (Calbiochem, San Diego, CA), LTD_4 and 5-HPETE (Biomol. Research Laboratories, Philadelphia, PA).

Results

Comparison of the cold-induced contractile responses in antigen-challenged and unchallenged isolated tracheal segments

Isolated tracheal segments obtained from normal (unsensitized) or OA-sensitized rats reacted with weak contractile responses (i.e., approximately 5% of ACh max.) to cold provocation (Figures 1 and 2). However, cold provocation (30°, 20° or 10°C) produced temperature-dependent, large and rapid contractile responses following recovery from antigen-induced responses (OA: 1 $\mu g\,ml^{-1}$) in the challenged sensitized segments (Figure 1–3).

Occasionally certain tissues which were obtained from sensitized animals failed to respond to antigen challenge (non-responders: Figure 1c) and the magnitude of the response to cold provocation was similar to that of the unchallenged sensitized or normal (unsensitized challenged) tracheal segments (Figures 1 and 2).

Effect of Ca^{2+} removal and verapamil on antigen-induced responses and subsequent development of airway hyperreactivity to cold

Incubation of rat isolated tracheal segments in Ca^{2+}-free Krebs-Henseleit solution produced substantial inhibition (82–90%) of antigen-induced contractions and subsequent development of airway hyperreactivity to cold (Figure 4a).

Verapamil (0.1–10 μM, 30 min preincubation) exerted concentration-dependent inhibition of antigen- and subsequent cold-induced responses with an IC_{50} of 0.88 and 1.9 μM, respectively. Verapamil (0.1–10 μM) did not exert any significant effect on the tissue contractility to ACh (10^{-3} M) (Figure 4b).

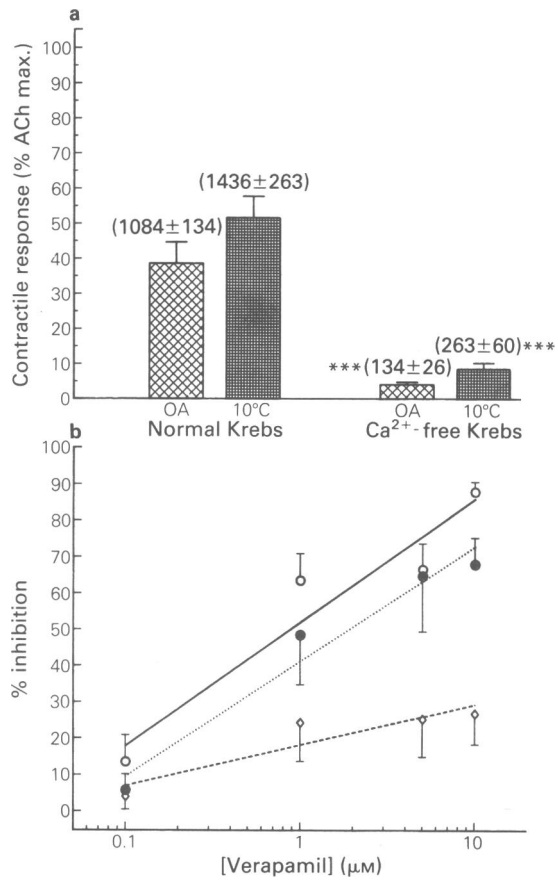

Figure 4 Inhibition of allergic responses (ovalbumin 1 $\mu g\,ml^{-1}$, 10–20 min) and subsequent development of airway hyperreactivity to cold provocation (10°C) by (a) Ca^{2+} removal from Krebs-Henseleit solution and (b) verapamil. Values are means with vertical lines indicating s.e.mean, (a) $n = 19$; (b) $n = 5–7$. In (a) $***P < 0.001$ indicates statistical significance determined by comparing allergic and cold responses in Ca^{2+}-free and Ca^{2+}-containing Krebs-Henseleit solutions (paired t test). In (a) numbers in parentheses above each column represent actual response in mg tension. (b) The inhibitory effects of verapamil (1, 5 and 10 μM, 30 min) on antigen (○)- and cold (●)-induced responses were statistically significant ($P < 0.05$; the ovalbumin and cold responses, % maximum response to acetylcholine (% ACh max.), in the absence and presence of each concentration of verapamil in each pair of tissues were compared by paired t test). For the ovalbumin response $IC_{50} = 0.881\,\mu$M and for the subsequent cold response $IC_{50} = 1.90\,\mu$M. (◇) Response to ACh, 10^{-3} M.

Since rat isolated tracheal segments obtained from actively sensitized rats exhibited remarkable airway hyperreactivity to cold provocation following recovery from allergic responses (Figures 1–3), these data led us to investigate the effect of antigen challenge on the reactivity of rat trachea to ACh, KCl and 5-HT. Rat isolated tracheal segments from normal or sensitized rats failed to respond to histamine (1–100 μM), leukotriene D_4 (0.1–1 μM) and 5-HPETE (0.1–1 μM). Cumulative concentration-effect curves to ACh, 5-HT and KCl in unchallenged segments and tracheal segments following recovery from OA-induced contractions were identical (data not shown). EC_{50}s of agonists in unchallenged tracheal segments were: ACh, 5.95 (3.85–9.19) μM; 5-HT, 1.46 (1.20–1.78) μM; KCl, 26.2 (24.52–28.19) mM, and in challenged tissues were ACh, 9.06 (6.43–12.77) μM; 5-HT, 1.42 (1.25–1.61) μM and KCl, 27.52 (25.61–29.57) mM.

Discussion

The inhibition of allergic responses and subsequent development of airway hyperreactivity to cold by removal of Ca^{2+} from Krebs-Henseleit solution, or by verapamil, suggest that the influx of Ca^{2+} from the extracellular medium into the airway smooth muscle is a prerequisite in the mediation of these responses. The data obtained in this study show that antigen (or endogenously released mediators), extracellular Ca^{2+} and cooling of the airways act synergistically to induce airway hyperreactivity to cold provocation. Ca^{2+}-channel blockers have been shown to provide a protective effect against EIA (Patel, 1981; Cerrina *et al.*, 1981; Corris *et al.*, 1983). The inhibitory effect of verapamil against EIA may be attributed to (i) the inhibition of Ca^{2+}-influx into the airway smooth muscle cells and (ii) the inhibition of Ca^{2+}-dependent step(s) in the synthesis and/or release of chemical mediators from tissue fixed mast cells/or other cell types.

Exposure of the airways to antigen (Weiss & Viswanath, 1974; this study), viral and bacterial infections, environmental pollutants, fog, cold etc., may result in an alteration in Ca^{2+} homeostasis (e.g., membrane permeability, decreased Ca^{2+} efflux, change in intracellular Ca^{2+} release and/or sequestration) in leukocytes, mast cells and other cells, and also in respiratory smooth muscle (Chand *et al.*, 1986b). The apparent increased Ca^{2+} permeability in respiratory smooth muscles following allergic reactions (Weiss & Viswanath, 1979; this study) may, at least in part, help to explain the mechanism of airway hyperreactivity to cold (exercise) provocation in asthmatics.

Some asthmatics are known to exhibit non-specific airway hyperreactivity to histamine, methacholine, prostaglandin $F_{2\alpha}$, leukotrienes, cold, fog, exercise, etc. (Skoogh, 1984). In rat trachea, the antigen-induced airway hyperreactivity to cold provocation appears to be a specific phenomenon because the concentration-effect curves of acetylcholine, KCl, 5-HT in challenged (following recovery from allergic responses) and unchallenged tissues were identical. Therefore, antigen-induced airway hyperreactivity to cold in rat trachea may not exactly relate to non-specific bronchial hyperreactivity in asthmatics.

It has been postulated that during exercise inhalation of cold dry air in asthmatics may increase osmolality in the airway mucosa and this could trigger bronchoconstriction (Anderson, 1984). In rat trachea, the increase in osmolality by D-glucose (100 mOsm), or D-mannitol (100 mOsm) was found to attenuate tissue contractility and did not enhance airway hyperreactivity to cold (unpublished observations). Although the actual underlying cause of bronchial hyperreactivity in asthmatics and exercise-induced asthma is still unknown, it would be of great interest to examine the effect of antigen and platelet-activating factor (Paf) on cold responses in airway smooth muscles of normal and allergic individuals. However, slight induction of tone in the airways by threshold concentrations of antigen and/or mediators (Paf, acetylcholine, 5-HT, KCl) seem to constitute an essential prerequisite for the induction of airway hyperreactivity to cold (Chand *et al.*, 1986a). The mediators(s), extracellular Ca^{2+} and cold act synergistically to induce hyper-responsiveness of respiratory smooth muscle of rats to cold provocation (Chand *et al.*, 1986a; this study).

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