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Relationship between Intracellular Calcium and Airway Reactivity in Guinea Pigs

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Abstract: The present study was carried out to examine the relationship between intracellular free calcium ion concentrations and its regulatory enzymes, sodium potassium adenosine triphosphatase (Na^+,K^+-ATPase) and calcium adenosine triphosphatase (Ca^{2+}-ATPase), with airway reactivity to inhaled histamine in guinea pigs. Forty-nine guinea pigs were included in this study. Of these, 34 animals responded to histamine bronchoprovocation challenge *in vivo* with a greater than 35% fall in specific airways conductance and were labeled as "reactive," and the remaining 15 were "nonreactive." The dose of histamine producing a 35% fall in specific airways conductance was labeled as ED_{35} SGaw. The animals were then sacrificed, and the following biochemical measurements were carried out: intracellular free calcium ion concentrations $[Ca^{2+}]_i$ in leukocytes and isolated tracheal smooth muscle cells, activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase in tracheal homogenate, and plasma levels of lysophosphatidylcholine (LPC). Reactive guinea pigs showed significantly higher $[Ca^{2+}]_i$ and Na^+,K^+-ATPase and Ca^{2+}-ATPase activities. Airway reactivity (ED_{35} SGaw) had significant negative correlation with $[Ca^{2+}]_i$, with activities of each of the ATPases and with plasma lysophosphatidylcholine. It is concluded that the level of $[Ca^{2+}]_i$ is an important determinant of airway reactivity. Intracellular calcium levels modulate airway response to histamine with higher levels being associated with greater reactivity. [Japanese Journal of Physiology, 51, 577–583, 2001]

Key words: airway hyperreactivity, intracellular calcium, Na^+,K^+-ATPase, Ca^{2+}-ATPase, lysophosphatidylcholine.

The regulation of intracellular calcium may play a crucial role in the pathogenesis of asthma because an increase in $[Ca^{2+}]_i$ is an essential initiating mechanism of cellular activation in inflammatory cells and in airway smooth muscle [1–4]. Because of the inaccessibility of tracheal tissue in human subjects, animal models have been used to define the biochemical correlates of airway reactivity. Na^+,K^+-ATPase and Ca^{2+}-ATPase are key enzymes involved in the regulation of $[Ca^{2+}]_i$. From our Institute, Nath *et al.* [5] observed a positive correlation between *in vivo* airway sensitivity to histamine and tracheal Na^+,K^+-ATPase and Ca^{2+}-ATPase activities in guinea pigs. Because these enzymes serve to lower the intracellular calcium, it was suggested that the higher activities in the more-reactive animals might reflect a compensatory response to higher $[Ca^{2+}]_i$ level. However, measurements of intracellular calcium were not made. The present study was carried out to examine the relationship between airway reactivity and intracellular calcium.

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; SGaw, specific airway conductance; ED_{35} histamine, dose of histamine that produced a fall of 35% in SGaw; LPC, lysophosphatidylcholine.

MATERIALS AND METHODS

Chemicals and reagents. Histamine, Tris, HEPES, ionomycin, EGTA, collagenase, protease, bovine serum albumin, and ouabain were obtained from Sigma, St. Louis, USA. Fura-2AM was bought from Molecular Probes Inc., USA. All other chemicals and reagents were locally available laboratory grade chemicals.

Animals. Specific pathogen-free wild-type guinea pigs of either sex, weight ranging from 250 to 650 g were used in this study. The animals were housed in groups of 6–8 in climate-controlled animal quarters in the animal house of the institute and were given water and food ad libitum. The study was approved by the University Review Committee.

Measurement of airway function. Measurements of specific airway conductance (SGaw) and airway reactivity to inhaled histamine were made in nonanesthetized, spontaneously breathing animals, using a noninvasive body plethysmographic technique as described previously [6]. Records of airflow changes were measured by using a Hans-Rudolf 0000 pneumatachograph and a transducer (Validyne MP-45 $\pm$2 cmH_2O) connected to an amplifier (Validyne CD12), and box pressure changes were measured by using a transducer (Validyne MP-45 $\pm$2 cmH_2O) connected to an amplifier (Validyne CD15). The outputs of the two amplifiers were fed into the *Y*- and *X*-channels of a Hewlett-Packard 54603B storage oscilloscope. A continuous plot of airflow change versus box pressure change was obtained with airflow on the *Y*-axis and box pressure on the *X*-axis. The inspiratory limb of the *X*–*Y* plot represented the ratio of flow change to flow-related changes in box pressure and provided the data for computing SGaw [6]. The slope of the rising limb of this *X*–*Y* loop was recorded in terms of tan θ that was obtained by dividing the voltage change on *Y*-axis by the voltage change on *X*-axis. SGaw at functional residual capacity (FRC) was calculated by substituting the value of tan θ in the following equation [6]:

$$\text{SGaw at FRC} = \tan\theta \times \{(\text{ml/s/div. on the ordinate}) / (\text{ml/div. on the abscissa})\} \times \{1/(P_B - P_{H_2O})\}$$

where P_B=atmospheric pressure, P_{H_2O}=water vapour pressure at ambient temperature.

Assessment of airway reactivity to histamine. Histamine aerosol was generated by passing an air current at 15 p.s.i. through a deVilbiss 645 nebulizer. Initially, phosphate buffered saline (PBS) was given in the form of aerosol for 30 s and SGaw measured 30 and 90 s post inhalation. The 90 s recording of SGaw was considered to be the control value. Subsequently, starting from 0.02 mg/ml, consecutively doubled concentrations of histamine in PBS were given, measuring the response to each at 30 and 90 s until SGaw fell by >35% or until a concentration of 2.5 mg/ml was reached. A log dose response curve was plotted, and the concentration of histamine producing a 35% fall in SGaw was calculated (ED_{35} histamine) as described by Agrawal [6]. This represented the airway reactivity of the animal. Animals that responded to inhaled histamine with a 35% or greater fall in SGaw were labeled as reactive, and the nonresponders, even at the highest dose of histamine, were referred to as nonreactive guinea pigs.

Isolation of tracheal smooth muscle cells. Guinea pigs were put under deep anesthesia by giving 50 mg/kg of pentothal sodium intraperitoneally. The isolation of tracheal smooth muscle cells was done by enzymatic digestion, as described [7–9], with slight modifications. Briefly, the tracheae were quickly dissected, placed in physiological salt solution (pH=7.4) on ice, and cleaned of the surrounding connective tissue under a Zeiss dissecting microscope. The tracheal smooth muscle was dissected free from the cartilage and incubated at 37°C in 2 ml of Ca^{2+}-free HEPES buffer containing 1.5 mg/ml collagenase Type I and 0.1 mg/ml protease for 15 min. The strip of tracheal smooth muscle was then washed in Ca^{2+}-free HEPES buffer and agitated vigorously by drawing it in and out of a Pasteur pipette to separate the free cells from undigested tissue. The undigested tissue was then minced into small pieces and incubated again at 37°C in Ca^{2+}-free HEPES buffer containing 1.5 mg/ml collagenase for 20 min. Protease was not added to minimize damage to free cells. The enzyme solution was discarded, and smooth muscle cells were dispersed to form a uniform cell suspension by vigorous agitation through a Pasteur pipette. The cells were washed twice in Ca^{2+}-free HEPES buffer by centrifuging at 100$\times g$ for 5 min to remove all traces of the enzyme, then resuspended in HEPES buffer with calcium.

After cell isolation in calcium free media, nearly 60% of the cells were rod shaped or oblong. In buffer containing calcium, however, almost all cells assumed a spherical shape within a few minutes. The viability after isolation was >85% as assumed by trypan blue exclusion [10] under a light microscope.

Separation of leukocytes from blood. Leukocytes were separated from heparinized whole venous blood [11]. Plasma was separated by centrifuging the blood at 200$\times g$ for 10 min. One ml of the plasma was kept separately at −20°C for phospholipid analysis.

Leukocytes were separated by dextran sedimentation of erythrocytes. Viability of the isolated cells was tested by trypan blue exclusion [10], and it was found to be greater than 95%. The cell pellet was suspended in Tris buffer (100 mM, pH=7.4) for ATPase estimation or HEPES buffer with composition as 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM HEPES adjusted to pH=7.4 with NaOH for $[Ca^{2+}]_i$ estimation.

Estimation of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities. These were measured in homogenized tracheal tissue. The tracheal tissue was dissected free of the surrounding connective tissue, minced into small pieces, and homogenized at 4°C by using a glass homogenizer. The undisrupted tissue fragments were sedimented by low-speed centrifugation at 150×*g* and the supernatant was used for ATPase assay.

ATPase activities were estimated according to the method of Schmalzing and Kutschera [12]. Cleavage of ATP was measured in a total volume of 0.5 ml in the presence of 5 mM $MgCl_2$, 2 mM Na_2ATP, 100 mM NaCl, and 100 mM Tris, pH=7.4). To this basal medium the following agents were added: (i) for Mg^{2+}-ATPase activity: 0.1 mM ouabain+2 mM EGTA; (ii) for Mg^{2+},Na^+,K^+-ATPase activity: 20 mM KCl+2 mM EGTA; and (iii) for Mg^{2+},Ca^{2+}-ATPase activity: 0.1 mM ouabain+150 μM $CaCl_2$. The pH values of the media were adjusted to 7.4 at 37°C. After temperature adjustment of the ATPase media, the ATPase reactions were initiated by the addition of the cell homogenate suspension (~100 μg protein). The reaction tubes were incubated at 37°C for 30 min. The reaction was stopped by the addition of ice-cold 500 g/*l* trichloroacetic acid (final concentration=50 g/*l*) followed by centrifugation at 18,000×*g* for 3 min at 0°C to remove proteins. A control set was run simultaneously, in which the enzyme was added after the reaction.

Protein was estimated by the method of Lowry *et al.* [13] with bovine serum albumin as a standard. The P_i liberated in the ATPase reaction was estimated [14] in the protein-free supernatant. The activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase were calculated by the difference between the quantity of P_i liberated in Mg^{2+},Na^+,K^+-ATPase and Mg^{2+}-ATPase activity, and in Mg^{2+},Ca^{2+}-ATPase and Mg^{2+}-ATPase activity, respectively. The results were expressed as μg P_i liberated/mg protein/30 min.

Measurement of leukocyte $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by using the fluorescent dye FURA-2AM, as described by Grynkiewicz [15]. Briefly, 2×10^6 leukocytes were loaded with FURA-2AM by incubating them with 2 μl of 1 mmol/*l* stock of dye in DMSO to a final concentration of 2 μM for 45 min at 37°C. The cells were then washed three times with HEPES buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM D-glucose, and 10 mM HEPES, pH=7.4) by centrifugation at 400×*g* for 2 min. The cells were then suspended in 2 ml of buffer (1×10^6 cells/ml). Fluorescence measurement was done in a Shimadzu spectrofluorometer Model RF-5000 at alternate excitation wavelengths of 340±10 and 380±10 nm and with emission wavelength of 510±20 nm at room temperature. The cells were continuously stirred with a magnetic stirrer throughout the experiment. The $[Ca^{2+}]_i$ was calculated by using the ratio method with the following equation: $[Ca^{2+}]_i=K_d(R-R_{min})/(R-R_{max})\times Sf_2/Sb_2$, where K_d represents dissociation constant of FURA-2 for $[Ca^{2+}]$, 224 nM at 37°C; R represents the ratio of fluorescence for the intracellular indicator at the two excitation wavelengths; R_{max} represents the ratio of maximum fluorescence, obtained after the addition of 10 μM ionomycin; R_{min} represents the ratio of minimum fluorescence, obtained after chelating all the $[Ca^{2+}]_i$ with EGTA; and Sf_2/Sb_2 represents the proportionality constants for the fluorescence of Ca^{2+}-free and Ca^{2+}-bound forms of the indicator at 380 nm, respectively.

Tracheal smooth muscle cells were isolated as described above, and $[Ca^{2+}]_i$ was estimated in tracheal cell suspension by the same method.

Estimation of plasma LPC. Plasma phospholipids were extracted according to the method of Phillips and Dodge [16]. Briefly, to 1 volume of plasma, 7 volumes of methanol were added, and the mixture was allowed to stand for 30 min at 4°C. Fourteen volumes of chloroform were then added and kept at 4°C with intermittent shaking. The mixture was filtered through Whatman filter paper No. 1 and emulsified with 1/5th the volume of normal saline, agitated vigorously, and allowed to stand at room temperature until the two layers separated. The upper layer was discarded, and the lower layer was evaporated to dryness in a rotary vacuum evaporator at 40°C. The residue was again dissolved in a known volume of chloroform. The quantitative analysis of the phospholipids extracted was done by the method of Skipski *et al.* [17] by using thin-layer chromatography. The P_i liberated was estimated by Bartlett's method [18]. Phospholipid fractions were expressed as a percentage of the total phospholipids.

Statistical analysis. Correlations between variables are expressed in terms of Pearson's or Spearman's coefficients of correlation, depending on

whether the distribution was normal or not. Between-group comparisons were done by applying Student's *t*-test. A p value of 0.05 or less was considered significant.

RESULTS

Forty-nine guinea pigs were included in this study. Of these, 34 responded to histamine challenge with a fall in SGaw of more than 35% (reactives). The ED_{35} ranged from 0.06 to 2.5 mg/ml histamine with a median value of 0.6 mg/ml. The remaining 15 animals were nonreactive to inhaled histamine.

Intracellular calcium levels (mean±SEM, nmol/*l*) are shown in Fig. 1. In leukocytes, the levels of $[Ca^{2+}]_i$ in reactive ($n=14$) and nonreactive ($n=6$) guinea pigs were 184.9±29.2 and 97.3±16.4, respectively ($p<0.05$). $[Ca^{2+}]_i$ in tracheal smooth muscle cells of reactive guinea pigs ($n=11$) was also greater than in nonreactive guinea pigs ($n=7$), but the difference was not statistically significant (149.5±27.1 and 107.3±9.7, respectively, $p>0.05$). A significant positive correlation between leukocyte and tracheal $[Ca^{2+}]_i$ levels was observed ($n=15$, $r=0.88$, $p<0.0001$)

A significant negative correlation between ED_{35} histamine and $[Ca^{2+}]_i$, was found in both leukocytes ($n=14$, $r=-0.77$, $p<0.0001$) and in tracheal smooth muscle cells ($n=11$, $r=-0.48$, $p<0.05$) (Fig. 2). The activities of both Na^+,K^+-ATPase and Ca^{2+}-ATPase (expressed as μg of P_i $30\,min^{-1}\,mg^{-1}$ of protein at 37°C) were found to be increased in reactives in comparison with to nonreactive guinea pigs (Fig. 3). Na^+,K^+-ATPase activity was 5.02±0.8 in the reactive animals ($n=24$) and 2.82±0.3 in nonreactive animals ($n=10$) ($p<0.05$). Ca^{2+}-ATPase activity was 3.47±0.6 in reactive guinea pigs ($n=24$) and 1.26±0.2 in nonreactive guinea pigs ($n=10$) ($p<0.01$). There was a significant positive correlation between Na^+,K^+-ATPase and Ca^{2+}-ATPase activities ($n=33$, $r=0.88$, $p<0.0001$).

The relationship between tracheal ATPase activities and airway reactivity is shown in Fig. 4. A significant negative correlation was observed between airway reactivity and tracheal Na^+,K^+-ATPase activity

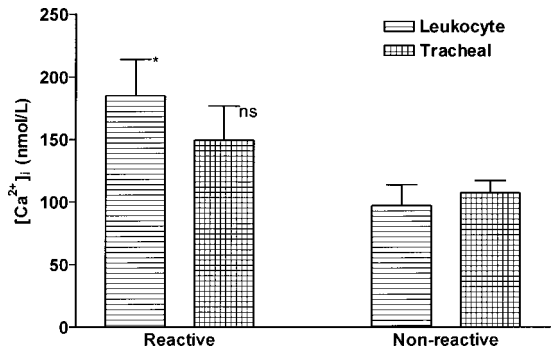

Fig. 1. Mean±SEM of $[Ca^{2+}]_i$ levels in leukocytes and tracheal smooth muscle cells of reactive and nonreactive guinea pigs. * $p<0.05$; ns not significant, $p>0.05$.

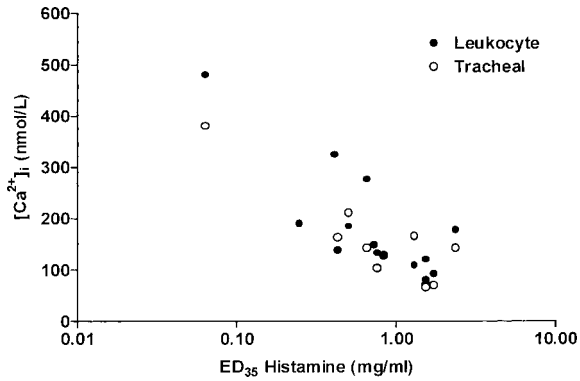

Fig. 2. Relationship between $[Ca^{2+}]_i$ and airway reactivity (expressed as ED_{35} histamine). $r=-0.77$, $p<0.0001$ for leukocytes; $r=-0.48$, $p<0.05$ for tracheal smooth muscle cells.

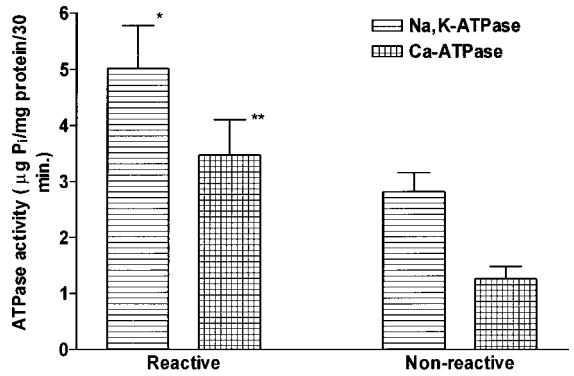

Fig. 3. Mean±SEM of Na^+,K^+-ATPase and Ca^{2+}-ATPase in tracheal tissue of reactive and nonreactive guinea pigs. * $p<0.05$, ** $p<0.01$.

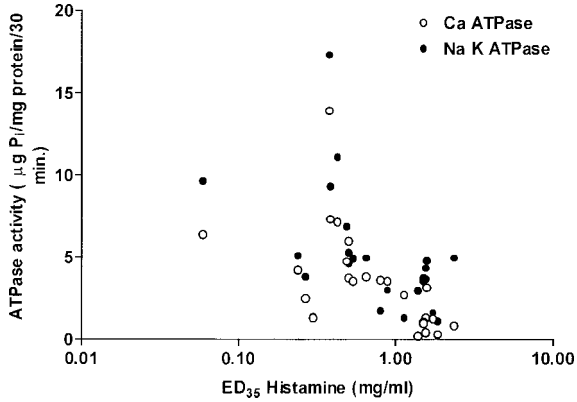

Fig. 4. Relationship between tracheal ATPase activity and airway reactivity. $r=-0.5$, $p<0.01$ for Na^+,K^+-ATPase and $r=-0.73$, $p<0.0001$ for Ca^{2+}-ATPase.

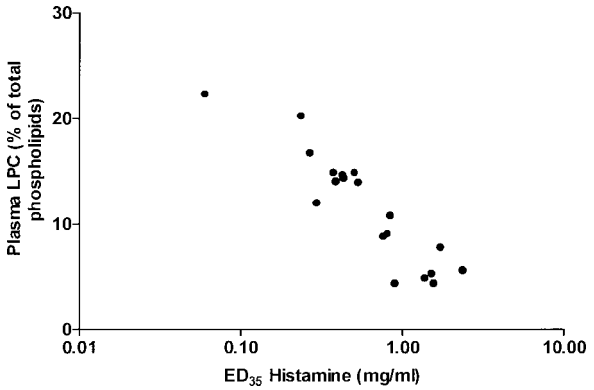

Fig. 5. Relationship between plasma LPC levels and airway reactivity. $r=-0.9$, $p<0.001$.

($r=-0.50$, $p<0.01$) and between airway reactivity and Ca^{2+}-ATPase activities ($r=-0.73$, $p<0.0001$).

The relationship between tracheal Ca^{2+}-ATPase and free intracellular calcium in tracheal smooth muscle cells was statistically not significant ($n=9$, $r=0.45$, $p>0.05$). Tracheal Na^+,K^+-ATPase, however, showed a significant positive correlation with tracheal $[Ca^{2+}]_i$ ($n=9$, $r=0.70$, $p<0.05$).

The mean±SEM value of plasma LPC (expressed as % of total phospholipids) in reactive animals ($n=19$) was found to be significantly higher than the values in nonreactive animals ($n=3$) (11.49±1.2 and 4.13±0.6, respectively, $p<0.001$). Among the reactive animals, a significant relationship was observed between the plasma LPC levels and airway reactivity ($n=19$, $r=-0.90$, $p<0.001$) (Fig. 5). Plasma LPC levels showed a significant positive correlation with tracheal Ca^{2+}-ATPase ($n=18$, $r=0.57$, $p<0.05$) and with tracheal Na^+,K^+-ATPase activity ($n=18$, $r=0.53$, $p<0.05$).

DISCUSSION

The present study demonstrates a significant relationship of airway reactivity with intracellular calcium ion concentration, Ca^{2+}-ATPase and Na^+,K^+-ATPase activities, and plasma LPC levels. These results also confirm the earlier report of Nath *et al.* [5] from our laboratory, who found a correlation between airway reactivity *in vivo* and tracheal Ca^{2+}-ATPase and between Na^+,K^+-ATPase activities and LPC levels. They, did not measure intracellular calcium, however. As a result of these observations, it was suggested that higher LPC levels resulted in greater membrane permeability, leading to Ca^{2+} influx and higher intracellular levels of calcium ions, which would activate Na^+,K^+-ATPase and Ca^{2+}-ATPase as a compensatory homeostatic response to lower $[Ca^{2+}]_i$. We observed, however, that in spite of higher activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase, the levels of $[Ca^{2+}]_i$ in reactive animals were significantly higher than in the nonreactive animals. This possibly reflected a new equilibrium for higher calcium levels in reactive animals. There was also a good correlation between leukocyte and isolated tracheal smooth muscle cell $[Ca^{2+}]_i$ levels, confirming previous reports that leukocyte can be used as a model cell system for the study of asthma [19]. We have recently reported increased $[Ca^{2+}]_i$ levels in leukocytes of asthmatic patients correlating with the severity of their affliction [20]. Previous studies have reported an elevated intracellular-free calcium ion concentration in the platelets of asthmatics [21]. Higher $[Ca^{2+}]_i$ could make the cells more responsive to any stimulus because it would be closer to the threshold level required for cell activation. This may explain the significant relationship between airway reactivity and $[Ca^{2+}]_i$, with animals having higher $[Ca^{2+}]_i$ being more reactive to histamine.

Reactive guinea pigs also showed a corresponding increase in ATPase activities. This increase may be a compensatory response to increased $[Ca^{2+}]_i$. The intracellular calcium levels in the present study were measured under basal conditions, i.e., not under condition of any stimulation. The airway response to histamine is a transient bronchoconstriction from which the animal recovers quickly. The animals had recovered from histamine-induced bronchospasm much before these were sacrificed for biochemical studies. Any effect of histamine on intracellular calcium would have disappeared by then. Therefore histamine inhalation is not very likely to have influenced the biochemical changes observed.

Calcium ions (Ca^{2+}) have been implicated in the activation of a variety of cell types. Calcium signaling is involved in such diverse processes as secretion, muscle contraction, cell growth and differentiation, and chemotaxis [22]. Thus the regulation of intracellular calcium ion concentration $[Ca^{2+}]_i$ is fundamental to the homeostasis of virtually all cells. Our study shows that increased airway reactivity is associated with a higher $[Ca^{2+}]_i$ level, emphasizing its role as a modulator of airway response. Besides an altered release from intracellular stores and entry across the cell membrane, the sensitivity of the contractile apparatus of the airway smooth muscle needs to be examined to explain this association.

Another important observation in the present study was a significant correlation between airway reactivity and plasma LPC levels, as well as significantly greater LPC levels in reactive guinea pigs in comparison with nonreactive animals. Plasma LPC also had a signifi-

cant direct correlation with tracheal Ca^{2+}-ATPase and Na^+,K^+-ATPase activities. LPC has long been known to be a cytolytic agent that increases ion permeability of the membranes [23, 24]. Thus LPC may directly increase calcium influx through the plasma membrane, as shown by Yu *et al.* [25]. Another mechanism for increasing the levels of free $[Ca^{2+}]_i$ could be a decrease in the levels of cAMP inside the cell. LPC has been shown to inhibit adenylate cyclase [26, 27] and to stimulate phosphodiesterase [28]. Thus LPC may be an important modulator of $[Ca^{2+}]_i$. We did not measure cAMP levels or activities of adenylate cyclase or phosphodiesterase. It has been documented earlier however, that reduced activity of adenylate cyclase is associated with increased airway reactivity [29]. Another possible mechanism by which LPC may alter airway reactivity is by the impairment of nitric oxide relaxation pathways [30].

We have earlier reported increased intracellular calcium in leukocytes of patients with asthma [20]. However, there are important differences between that study and the present one. We have found that as the airway reactivity increases, the activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase also increase. In contrast, in the earlier study asthmatics with a more severe disease had a greater decrease in the activities of these ATPases. The reason for these contrasting observations may be that the animals in the present study were not sensitized and had no natural or experimental asthma. The decreased activities of the ATPases may play an important role in the pathogenesis of asthma [20, 31]. But in both studies the importance of increased intracellular calcium as a modulator of airway responses has been revealed.

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