Evidence for an Increased Intracellular Free Calcium Concentration in Platelets of Bronchial Asthma Patients

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The pathogenesis of bronchial asthma is not yet fully understood. Recently much attention has been given to the hypothesis that intracellular free calcium ($[Ca^{2+}]_i$) metabolism is abnormal in various diseases. In this study we investigated whether $[Ca^{2+}]_i$ exists abnormally in subjects with bronchial asthma. The $[Ca^{2+}]_i$ in 32 treated or untreated subjects with bronchial asthma were compared with 63 normal subjects. Resting levels of $[Ca^{2+}]_i$ were estimated by loading the fluorescent indicator Fura-2 in washed platelets. The $[Ca^{2+}]_i$ level in the control subjects was 129.7 ± 18.0 nM (mean $\pm$ SD). However, in that of the bronchial asthma patients was 152.7 ± 44.1 nM, significantly higher than that of the control subjects ($p < 0.05$). It is well recognized that an increase of $[Ca^{2+}]_i$ in vascular smooth muscle involves contraction. The findings suggest that the same phenomenon is quite possible in the tracheal smooth muscle and that it plays an important role in the pathogenesis of bronchial asthma.

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Key words: Ca^{2+} influx, Fura-2, bronchial constriction

Introduction

Bronchial asthma is characterized by reversible airway obstruction which is induced by the constriction of bronchial smooth muscle, secretion of mucus and mucosal edema (1). The important role of calcium ions in the regulation of skeletal muscle construction was established in 1970s by Ebashi (2). Since then, $[Ca^{2+}]_i$ levels have been used as a reliable indicator of smooth muscle constriction. The relationship between $[Ca^{2+}]_i$ and muscle tension has been investigated by radioactive $^{45}Ca^{2+}$ flux experiments. In general, there is a good correlation between the ratio of $^{45}Ca^{2+}$ influx and smooth muscle constriction (3–6). However, it is difficult to directly measure $[Ca^{2+}]_i$ in patients by this method. In the 1980s, Tsien synthesized a new intracellular Ca^{2+} indicator, Quin2, which was followed by the improved indicators, Fura-2 and indo-1 (7). At present, these indicators are widely used to examine the changes of $[Ca^{2+}]_i$ in hypertensive study. To our knowledge, measurement of $[Ca^{2+}]_i$ in patients with bronchial asthma using this method has not been previously reported. In this study we measured $[Ca^{2+}]_i$ directly in platelets obtained from patients with bron-

chial asthma using a fluorescence method.

Materials and Methods

Subjects: 32 (14 males; 18 females) treated or untreated patients with bronchial asthma and 63 (37 males; 26 females) healthy control subjects were investigated in this study. The mean age of asthmatic patients was 49 ± 21 SD in males and 38 ± 21 SD in females. On the other hand, the mean age of control subjects was 39 ± 17 SD in males and 44 ± 17 SD in females.

Preparation of platelet-rich plasma (PRP)

Figures 1 and 2 schematically illustrate the whole procedure and the device for measuring the $[Ca^{2+}]_i$. Venous blood (9ml) was collected with a 21 gauge needle. Blood was drawn into a polypropylene syringe containing 1 ml of 3.8% sodium citrate as the anticoagulant. When we collect the blood sample, we usually release the tourniquet and take it slowly and steadily for a few minutes in order to prevent activation of platelets (8). After drawing a 9 ml blood sample, a second syringe was

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Figure 1. Measurement method of intracellular free calcium concentration.

Figure 2. Measuring device of intracellular free calcium concentration. M: concave mirror, SO: Xe lamp, DM1, DM2: dichroic mirror, SH: shutter, F1: interference filter (340nm), F2: interference filter (380nm), L1, L2, L3, L4: lens, BD: beam diaphragm, F3: interference filter (500nm), S: sample, PM: photomultiplier tube, C: computer, DH: data-handling system.

connected and a 5 ml of blood sample was collected which was used for measuring the sodium, potassium, total calcium and magnesium in serum. The blood was immediately centrifuged at 800 rpm for 15 minutes at 20°C. The PRP was removed and the number of platelets was counted; citric acid was added and centrifuged at 2,600 rpm for 15 minutes at 20°C to prepare the platelet pellet. The platelet pellet was suspended in 10 mM N-

2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer containing 1 μM Fura-2-acetoxymethyl ester (Fura-2-AM) to a final concentration of 10⁵ cell/ μl and then incubated in a water bath for 45 minutes at 37°C. The platelet suspension was then placed on a 9×300 mm Sepharose CL-2B column which had been previously equilibrated with 100 ml of HEPES buffer. The platelet fraction in the elute, which was identified by

the appearance of turgidity, was collected in 1 ml vials and then 1 mM calcium chloride was added and incubated for 45 minutes at 37°C.

Estimation of $[Ca^{2+}]_i$

Fluorescence intensity was measured at an emission wavelength of 500 nm and sequential excitation wavelength of 340, 380 nm using a fluorometric spectrophotometer (CAF-100: Japan Spectroscopic Co. Ltd., Tokyo, Japan). Specimens were then lysed with 0.5% Triton X-100 (Sigma Chemical Co. Ltd., St. Louis, MO, USA), and the maximal ratio of fluorescence 340/380 was recorded. After 10 ml ethylene glycol-bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) was added, the minimal ratio of 340/380 was then measured. Using this ratio of 340/380, $[Ca^{2+}]_i$ was calculated by the following equation (9):

$$[Ca^{2+}]_i = Kd \times (R - R_{min} / R_{max} - R) \times \beta$$

where Kd represents the dissociation constant of Fura-2 for calcium (224 nM), R is the ratio of fluorescence at excitation wavelengths 340 and 380 nm in intact cell suspension, R_{max} and R_{min} are the ratio of fluorescence at 340 and 380 nm under calcium-saturated and calcium-free conditions, respectively, and β is the fluorescence ratio at 380 nm of R_{min} to R_{max} . R_{max} was determined by lysing the cell with 50 μ M digitonin in the presence of 1 mM calcium. R_{min} was then determined by the successive addition of 10 mM EGTA followed by adjustment of pH to 8.3 with 30 mM Tris.

Other biochemical analysis

Concentration of sodium and potassium in serum were measured by 480 Flame Photometer (Ciba-Corning Diagnostic Co. Ltd., Tokyo, Japan). Total measurement of calcium and magnesium were carried out by Ca-Mg fluorescent analyzing Meter 30/20 (Jookoo Co., Ltd., Tokyo, Japan). Serum IgE was measured by standard radioimmunoassay.

Statistical analysis

All data are presented as mean $\pm$ SD unless otherwise noted. Statistical comparisons were performed using unpaired Student's t-test with $p < 0.05$ considered as significant.

Results

Table 1 shows $[Ca^{2+}]_i$ concentrations in healthy normal subjects of different sexes and age groups. There were no significant differences observed in both sexes and between age groups. Thus we used the value of 129.7 ± 18.0 nM (combined 37 males and 26 females) as a mean control value. The deviation of this control data was about 14% indicating that our method can be considered accurate and reproducible.

Table 2 shows $[Ca^{2+}]_i$ concentrations in patients with bronchial asthma of sexes and of different age groups. There were no significant differences in the levels between sexes and the age groups, thus we compared the value of 152.7 ± 44.1 nM (combined 14 males and 18 females) to the control value. The $[Ca^{2+}]_i$ concentration in bronchial asthma patients was significantly

Table 1. The Concentrations of $[Ca^{2+}]_i$ in Healthy Normal Subjects of Different Sexes and Age Groups

Age	$[Ca^{2+}]_i$ (nM) in males	n	$[Ca^{2+}]_i$ (nM) in females	n	t-test
Under 30	130.3 $\pm$ 18.2	11	120.7 $\pm$ 16.5	6	NS
30-49	134.6 $\pm$ 18.2	16	129.3 $\pm$ 24.1	9	NS
50-69	130.8 $\pm$ 18.1	7	126.0 $\pm$ 17.1	9	NS
Over 70	135.0 $\pm$ 19.7	3	137.1 $\pm$ 4.8	2	NS
Total	131.8 $\pm$ 17.4	37	126.8 $\pm$ 18.5	26	NS

Table 2. The Concentrations of $[Ca^{2+}]_i$ in Bronchial Asthma Patients of Both Sexes and of Different Age Groups

Age	n	$[Ca^{2+}]_i$ (nM)	Statistical analysis
Under 30	11	142.1 $\pm$ 42.4	vs 30-49 NS vs Over 70 NS
30-49	10	156.3 $\pm$ 21.7	vs 50-69 NS vs Over 70 NS
50-69	6	163.8 $\pm$ 82.1	vs Over 70 NS
Over 70	5	155.8 $\pm$ 32.2	
Total	(m) 14 (f) 18	156.0 $\pm$ 53.3 150.2 $\pm$ 38.5	(f) vs (m) NS

Figure 3. Intracellular free calcium concentration in normal subjects and in bronchial asthma patients.

higher than that of normal subjects ($p < 0.05$), as shown in Fig. 3.

Serum calcium, sodium, potassium and magnesium in normal subjects and in bronchial asthma patients are summarized in Table 3. In each parameter a similar value was obtained and

no significant differences were observed the two groups. Figure 4 shows the relationship between $[Ca^{2+}]_i$ concentration and serum calcium (a), peripheral eosinophilic count (b) and serum IgE (c). No significant correlation was observed between $[Ca^{2+}]_i$ concentration and each parameter.

Discussion

In the last decade, much attention has been given to the hypothesis that cellular calcium metabolism is abnormal in hypertension (10, 11), cardiac diseases (12, 13), renal diseases (14, 15), lung diseases (3, 16), diabetes mellitus (17–20), secretion of various endocrine and exocrine hormones (21–26), preeclampsia (27), and platelet aggregation (28). The involvement of $[Ca^{2+}]_i$ in smooth muscle constriction and relaxation is a well recognized phenomenon, however the mechanisms by

Table 3. Serum Calcium, Sodium, Potassium and Magnesium in Normal Subjects and in Bronchial Asthma Patients

Serum components	Normal subjects	Patients with bronchial asthma	t-test
Ca (mg/dl)	10.2±1.2	10.2±1.2	NS
Na (mEq/l)	142.7±4.9	141.8±2.7	NS
K (mEq/l)	4.0±0.5	3.8±0.4	NS
Mg (mg/dl)	2.0±0.7	2.1±0.5	NS

Figure 4. Relationship between intracellular free calcium concentrations and serum calcium (a), eosinophil (b) and IgE (c) in bronchial asthma patients.

which changes in $[Ca^{2+}]_i$ concentrations within the cell are not clearly understood, particularly in the smooth muscle of the airway. In the second half of 1970s and the first half of 1980s several investigators have reported that an increase of $[Ca^{2+}]_i$ concentration activates not only respiratory smooth muscles, but also mast cell, bronchial mucus glands and the vagi. Calcium antagonists that block Ca^{2+} influx through specific Ca^{2+} channels in cytoplasmic membranes were therefore expected to be effective in the treatment of bronchial asthma (1, 4–6). However, these studies were performed using in vitro studies or animal studies, because it was difficult to measure the concentration of $[Ca^{2+}]_i$ directly in patients with bronchial asthma. In 1983, Tsien and his colleagues synthesized a new intracellular Ca^{2+} indicator, facilitating the direct measurement of $[Ca^{2+}]_i$ concentration in patients using the blood components.

In this study, we measured the concentration of $[Ca^{2+}]_i$ in patients with bronchial asthma and found that it was significantly higher than that in normal subjects. To our knowledge, measurement of the concentration of $[Ca^{2+}]_i$ in patients with bronchial asthma using the fluorescence method has not been previously reported. Although the mechanism of the increase of $[Ca^{2+}]_i$ concentration in bronchial asthma is not clear in the present study, calcium ion, when stimulated, is mobilized from intracellular sources such as the inner surface of plasma membrane and sarcoplasmic reticulum (16, 29) and from extracellular sources through transmembrane-specific Ca^{2+} channels (16, 30).

Although the pathophysiologic role of an increase of $[Ca^{2+}]_i$ concentration in bronchial asthma has not been fully clarified in the present study, it supports the fact that calcium ion plays an important role in allergic reactions (31–33). Principal pathogenetic features of bronchial asthma result from the activation and response of several different cell types in various combinations. Recent evidence demonstrates that these cell types all require calcium-dependent reactions for activation (5, 34–40). Therefore, it appears that the increased $[Ca^{2+}]_i$ concentration in patients with bronchial asthma, as demonstrated in the present study, may have been a calcium-dependent reaction inducing bronchospasm and/or causing mucosal edema and mucous plugging of airways mediated by the activation of several different cell types in various combinations. The cell types most commonly associated with airway obstruction are smooth muscle cells, mast cells, mucous gland secretory cells, vagal nerve cells and inflammatory cells.

On the other hand, several investigators reported that platelet-activating factor (PAF) plays an important role as a mediator in inflammatory and allergic disease of the lung (41, 42). PAF is secreted by platelets, macrophages and endothelial cells (43). The biological effects of PAF include release of toxic oxygen products, histamine, and metabolites of arachnoidic acid from neutrophils, eosinophils and tissue macrophages (44). PAF also increases the $[Ca^{2+}]_i$ concentration in macrophages (45). Moreover, it has been demonstrated that bradykinin which has been suggested to have involvement in the pathogenesis of allergic asthma can also induce an increase of the $[Ca^{2+}]_i$

concentration in both human (46, 47), and guinea-pig (48) airway smooth muscle cells. Therefore these inflammatory substances may also have been factors affecting the increase of $[Ca^{2+}]_i$ concentration in the present study.

Many studies (1, 4, 6, 31–33) demonstrated the effects of calcium antagonists on resting airway function. Most studies have failed to demonstrate any bronchodilator action of either nifedipine or verapamil. However, there are several reports (31–33). Of the effects of calcium antagonists on bronchoconstriction induced by various challenges such as exercise, histamine, methacholine and antigen. Although calcium antagonists do not appear to be very effective during short administration, it is possible that they may be more effective when given over an extended period of time, particularly if they have effects on airway mucus secretion, on bronchial mucosal edema or on inflammatory cell infiltration. This has not yet been adequately investigated.

In summary, we have described that the $[Ca^{2+}]_i$ concentration in patients with bronchial asthma is significantly higher than in normal subjects. This result indicates that an abnormal calcium metabolism in the cell may play an important role in the pathogenesis of bronchial asthma. It is furthermore necessary to investigate the relationship of abnormal calcium metabolism in platelets and bronchial asthma.

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