

A circulating inhibitor of the platelet Na^+/K^+ adenosine triphosphatase (ATPase) enzyme in allergy

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Previous investigations have documented a reduced activity of the sodium-potassium-stimulated adenosine triphosphatase enzyme (Na^+/K^+ ATPase) in platelet membranes of allergic subjects. The purpose of this study was to determine if the reduced Na^+/K^+ ATPase activity was due to an enzyme inhibitor. Na^+/K^+ ATPase activity of a particulate fraction of sonicated platelets was determined by spectrophotometry in asymptomatic adults with and without allergy. The Na^+/K^+ ATPase level (mean, nanomoles per microgram of protein per minute; $\pm$ STD) of allergic subjects (0.9 ± 1.3) was lower ($p < 0.001$) than that of nonallergic subjects (3.9 ± 1.6). In contrast, when the same platelet fractions were frozen before assay, Na^+/K^+ ATPase was higher ($p < 0.005$) in allergic subjects (6.0 ± 1.4) than in nonallergic subjects (3.6 ± 2.0). An inhibitor of canine kidney Na^+/K^+ ATPase was detected in the buffer in which these platelet fractions were frozen, allergic subjects ($0.5\% \pm 0.4\%$ inhibition per microgram of protein) compared to nonallergic subjects ($0.04\% \pm 0.08\%$; $p < 0.005$). The level of inhibition correlated positively with the postfreezing increase in platelet membrane Na^+/K^+ ATPase, suggesting a freezing-induced displacement of an inhibitor from the membrane. Plasma from these same subjects inhibited Na^+/K^+ ATPase activity of normal platelets, allergic subjects ($70\% \pm 31\%$ inhibition) compared to nonallergic subjects ($13\% \pm 16\%$; $p < 0.001$). These data suggest that the transport-enzyme defect observed in platelets from allergic subjects was due to a circulating Na^+/K^+ ATPase inhibitor. In vivo Na^+/K^+ ATPase inhibition in allergy could have profound effects on intracellular cation concentrations and broad implications for pathogenesis. (J ALLERGY CLIN IMMUNOL 1991;87:476-82.)

The biochemical defect responsible for allergy, a disease affecting up to 20% of the human population, is unknown. A defect involving sodium metabolism has been postulated.^{1,2} Moreover, a variety of circulating and noncirculating cell types, including the blood platelet,³ have been implicated in the pathogenesis of allergy.⁴⁻¹⁰ Previous investigations have documented a reduction of Na^+/K^+ ATPase enzyme activity in freshly isolated platelets of allergic patients.¹¹ The hypothesis that the reduced platelet Na^+/K^+ ATPase enzyme activity in allergy was due to an enzyme inhibitor was tested in the current study. The results demonstrate that the reduced enzyme ac-

Abbreviations used

ATP:	Adenosine triphosphate
ATPase:	Adenosine triphosphatase
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	Ethyleneglycol-bis-(β -aminoethylether)- N,N' -tetraacetic acid
NADH:	Reduced nicotinamide-adenine dinucleotide
Na^+ :	Sodium
K^+ :	Potassium
Ca^{++} :	Calcium
Mg^{++} :	Magnesium
STD:	Standard deviation

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tivity was due to an Na^+/K^+ ATPase inhibitor, which was displaced from the platelet membrane by the freezing process. Moreover, a dilutable Na^+/K^+ ATPase inhibitor with an extracellular binding site was also detected in the plasma of patients with allergy. These data provide evidence that Na^+/K^+ ATPase inhibition is associated with allergy and may play a role in pathogenesis.

METHODS

Three groups of adult subjects were identified, based on history, physical examination, and laboratory studies: (1) subjects with allergic rhinitis, defined by seasonal and/or perennial nasal symptoms and positive intradermal skin tests (>5 mm wheal) to pollens, molds, or dust (N = 18); nine of the subjects in this group also had a history of asthma, (2) potentially allergic subjects, defined by the absence of seasonal and/or perennial nasal symptoms but the presence of positive intradermal skin tests to pollens, molds, or dust (N = 5), and (3) normal individuals, defined by the absence of seasonal and/or perennial nasal symptoms and negative intradermal skin tests to pollens, molds, and dust (N = 10). A detailed description of the subjects has been reported previously.¹¹ On the study day, a venous blood sample was obtained for assay of prefreezing and postfreezing platelet Na⁺,K⁺-, Mg⁺⁺-, and Ca⁺⁺-stimulated ATPase activities, plasma ATPase-inhibitory activity, and total serum IgE levels by fluoroallergosorbent test (FAST).¹² Data were analyzed with Student's *t* test.

Platelet preparation and ATPase assay

Platelets were isolated, sonicated, and serially centrifuged, and the resultant particulate fraction was resuspended in 1 ml of Tris-sucrose as previously described.^{11,13} A portion of the particulate fraction was assayed in duplicate for ATPase enzyme activities within 2 hours of sample collection. The remainder of the platelet particulate fraction was frozen immediately at -70° C, thawed 1 to 4 weeks later, and centrifuged. The supernatants were assayed for Na⁺,K⁺ ATPase inhibitory activity, and the platelet membrane fractions were resuspended in Tris-sucrose and assayed for ATPase activities.

The prefreezing and postfreezing platelet particulate fractions were assayed for (Na⁺,K⁺), Mg⁺⁺, and Ca⁺⁺ ATPase activities at 37° C by the linked pyruvate kinase-lactate dehydrogenase, spectrophotometric method.¹⁴ The reaction mixtures for these assays and the method for calculating Na⁺,K⁺ ATPase activity have been described previously.¹¹ Protein content was assayed according to the method of Lowry et al.,¹⁵ and enzyme activities were expressed as nanomoles per microgram of protein per minute. The coefficient of variation was 10% for a single prefreezing sample assayed 11 times for Na⁺,K⁺ ATPase (intra-assay), 11% for a single postfreezing sample assayed 10 times in the same Na⁺,K⁺ ATPase assay (intra-assay), and 7% for a single postfreezing sample assayed 10 times in 10 different Na⁺,K⁺ ATPase assays (interassay). Data on the temporal reproducibility of the platelet measurements have been reported previously.¹¹

Platelet supernatant Na⁺,K⁺ ATPase inhibitory activity

The supernatant (250 µl) of centrifuged frozen membrane fragments of platelets derived from 1 ml of whole blood was combined with 250 µl of H₂O, and 500 µl of the reaction mixture described previously for Na⁺,K⁺ ATPase.¹¹ To initiate the enzyme reaction, canine kidney Na⁺,K⁺ ATPase enzyme (Sigma Chemical Co., St. Louis, Mo., specific activity, 1.1 U/mg of protein) was added (5 µl, concentra-

tion 0.15 mg of enzyme protein per milliliter of Tris buffer). The hydrolysis of ATP was measured spectrophotometrically as described.¹⁴ The percent inhibition was defined as the percent decrease from control activity in the presence of the platelet supernatant and expressed as percent inhibition per microgram of protein in the platelet membrane fraction.¹⁵ The coefficients of variation for a single sample assayed for Na⁺,K⁺ ATPase-inhibitory activity 10 times were 3% (intra-assay) and 4% (interassay) in the 80% inhibition range, and 8% (intra-assay) and 10% (interassay) in the 40% inhibition range.

Plasma Na⁺,K⁺ ATPase-inhibitory activity

Plasma was deproteinized by acidification to pH 5.5 with hydrochloric acid and boiling for 3 minutes.¹⁶ Platelets obtained from a normal human donor served as the ATPase enzyme source. Two milliliters of deproteinized plasma (pH 7.4) from the study subjects was incubated for 30 minutes at 37° C with the platelets obtained from 2 ml of the normal donor's plasma. The platelets were then separated by centrifugation, washed, sonicated, serially centrifuged, resuspended in Tris buffer, and assayed spectrophotometrically for ATPase activity as described above for the platelet ATPase assay. Freshly isolated platelets for these experiments were provided by the normal donor on six different occasions. The control ATPase activities of these six platelet samples ranged from 3.8 to 6.2 nmole/µg of protein per minute (mean, 5.0 ± 0.9) for Na⁺,K⁺ ATPase, 22.7 to 26.0 nmole/µg of protein per minute (mean, 24.6 ± 1.4) for Mg⁺⁺ ATPase, and 12.5 to 22.9 nmole/µg of protein per minute (mean, 19.3 ± 3.7) for Ca⁺⁺ ATPase. Five or six plasma samples from the study subjects were assayed each time that the platelets were obtained from the normal donor. The percent inhibition was defined as the percent decrease from control activity in the presence of the plasma.

Na⁺,K⁺ ATPase inhibitory activity in the serially diluted allergic plasma was assayed as described above for the platelet supernatant, except that (1) 500 µl of deproteinized plasma was substituted for 250 µl of the frozen platelet supernatant, (2) 250 µl of H₂O was deleted, and (3) 20 µl of the canine kidney Na⁺,K⁺ ATPase enzyme was used. The percent inhibition was defined as the percent decrease from control activity in the presence of the plasma.

RESULTS

The prefreezing and postfreezing platelet Na⁺,K⁺ ATPase-activity levels of allergic, potentially allergic, and normal subjects are illustrated in Fig. 1. Consistent, large, postfreezing increases in Na⁺,K⁺ ATPase activity were observed in the allergic and potentially allergic subjects, whereas increases of a lesser magnitude or decreases were observed for the normal subjects. The mean (±1 STD) prefreezing platelet Na⁺,K⁺ ATPase activities (nanomoles per microgram of protein per minute) of allergic subjects (0.9 ± 1.3) was significantly lower than that of normal subjects (3.9 ± 1.6; *p* < 0.001), whereas an intermediate value was observed for potentially allergic subjects

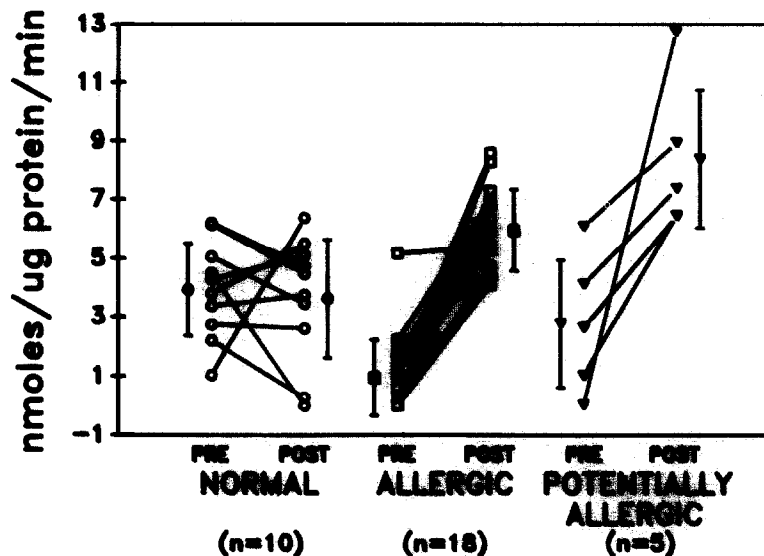


FIG. 1. Platelet Na^+, K^+ ATPase activities in allergic ($N = 18$), potentially allergic ($N = 5$), and normal subjects ($N = 10$). Vertical bars represent 1 SD above and below the mean value.

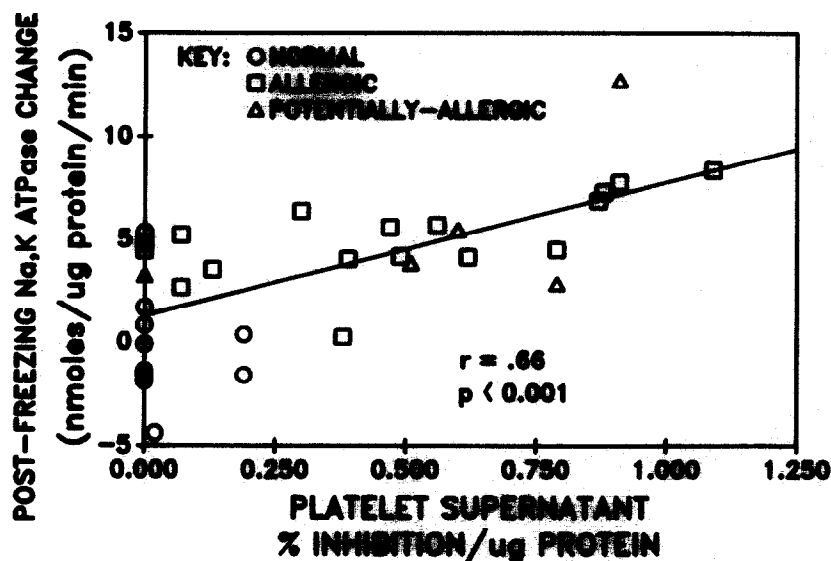


FIG. 2. Na^+, K^+ ATPase-inhibitory activity in the supernatant of frozen platelet membranes versus the freezing-induced change in platelet membrane Na^+, K^+ ATPase activity.

(2.8 ± 2.2). The opposite was observed for the same samples when samples were assayed after freezing: 6.0 ± 1.4 for allergic and 8.4 ± 2.4 for potentially allergic compared to normal subjects (3.6 ± 2.0 ; $p < 0.005$). The difference between the prefreezing and postfreezing samples was also significantly different between the groups, such that the freezing process was associated with a striking increase in Na^+, K^+ ATPase activities of allergic (5.0 ± 1.9) and potentially allergic subjects (5.6 ± 3.7) but a decrease in that of the normal subjects (-0.3 ± 2.5 ; $p < 0.001$ and $p < 0.005$, respectively).

In contrast, the prefreezing values and freezing-associated changes for Mg^{++} and Ca^{++} ATPases demonstrated a much greater degree of variability among the groups of subjects. The mean (± 1 STD) prefreezing and postfreezing Mg^{++} ATPase activities (nanomoles per microgram of protein per minute), respectively, were similar for allergic (23 ± 4 and 24 ± 6), potentially allergic (21 ± 7 and 23 ± 4), and normal subjects (22 ± 6 and 26 ± 3). The same was true for the Ca^{++} ATPase activities of allergic (11 ± 4 and 11 ± 6), potentially allergic (12 ± 4 and 11 ± 3), and normal subjects (13 ± 3 and

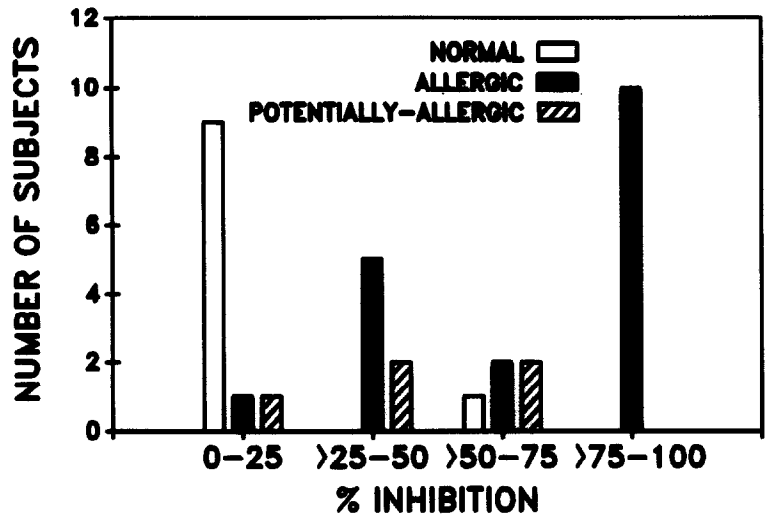


FIG. 3. Distribution of plasma Na⁺,K⁺ ATPase inhibitory activity in allergic, potentially allergic, and normal subjects.

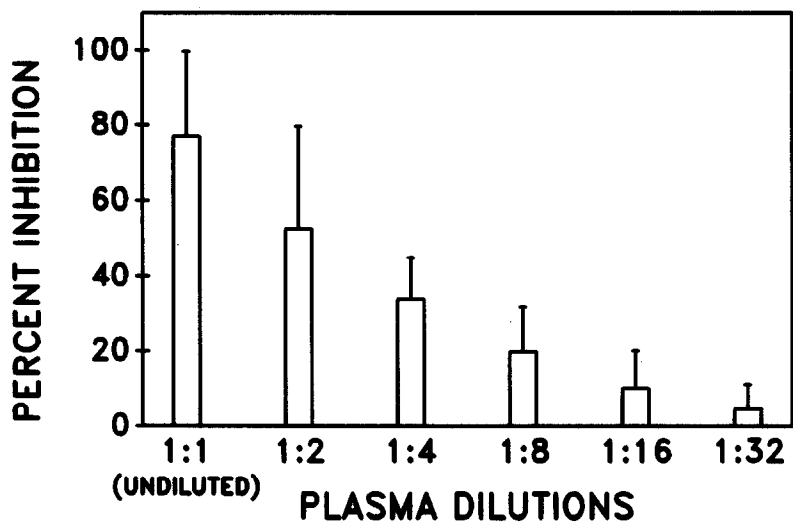


FIG. 4. Na⁺,K⁺ ATPase-inhibitory activity of serial dilutions of plasma from allergic subjects (N = 3).

7 ± 4). None of the differences between allergic, potentially allergic, and normal subjects were statistically significant.

The difference between prefreezing and postfreezing Na⁺,K⁺ ATPase activities was plotted against the corresponding prefreezing Na⁺,K⁺ ATPase value. A significant negative correlation was observed ($r = -0.76$; $p < 0.001$), such that platelet samples with low prefreezing levels of activity had large freezing-associated increases, and vice versa, for samples with high prefreezing activity levels.

To test the hypothesis that an enzyme inhibitor was present on freshly isolated platelet membranes and was displaced from the membrane during the freezing

process, the supernatant of the frozen platelet fractions was examined for its ability to inhibit canine kidney Na⁺,K⁺ ATPase. Significantly greater levels of inhibitory activity (mean percent inhibition of canine kidney Na⁺,K⁺ ATPase per microgram of protein; ± 1 STD) were observed in the supernatants of platelets from allergic (0.5 ± 0.4) and potentially allergic (0.6 ± 0.3) subjects compared to that of normal subjects (0.04 ± 0.08; $p < 0.005$). Moreover, the level of inhibitory activity in the platelet supernatant correlated positively with the freezing-associated increase in platelet Na⁺,K⁺ ATPase activity (Fig. 2).

To determine if a circulating ATPase enzyme inhibitor was present in allergic subjects, plasma was

examined for its ability to inhibit the ATPase activities of platelets derived from a normal subject. The distribution of the plasma Na^+/K^+ ATPase-inhibitory activity for the 33 subjects is illustrated in Fig. 3. Low levels of plasma-inhibitory activity were detected in nine of 10 normal subjects, whereas higher levels were detected in most allergic and potentially allergic subjects. The mean values (percent inhibition of control platelet Na^+/K^+ ATPase activity, ± 1 STD) for the allergic (70 ± 31) and potentially allergic subjects (40 ± 23) were significantly different from that of the normal subjects (13 ± 16 ; $p < 0.001$ and $p < 0.025$, respectively). Moreover, the level of plasma Na^+/K^+ ATPase-inhibitory activity correlated negatively with the prefreezing Na^+/K^+ ATPase activity of corresponding platelets ($r = -0.44$; $p < 0.01$). As illustrated in Fig. 4, the Na^+/K^+ ATPase-inhibitory activity of plasma from three allergic subjects was dilutable.

In contrast, the levels of plasma-inhibitory activity were highly variable for the platelet membrane Mg^{++} and Ca^{++} ATPase activities. The percent inhibition by the plasma of allergic, potentially allergic, and normal subjects, respectively, was 9 ± 15 , 8 ± 6 , and 7 ± 8 for Mg^{++} ATPase; and 26 ± 23 , 20 ± 18 , and 12 ± 17 for Ca^{++} ATPase. None of the differences among the three groups was statistically significant. Correlations between the level of plasma-inhibitory activity and the prefreezing ATPase activity of corresponding platelets were not observed for Mg^{++} and Ca^{++} ATPases.

The subjects were then grouped according to total serum IgE concentration, and mean values (± 1 STD) for the Na^+/K^+ ATPase parameters were calculated and compared. Compared to the subjects with low serum IgE concentrations, subjects with high serum IgE had significant reductions in prefreezing platelet Na^+/K^+ ATPase activity (1.5 ± 1.8 versus 3.2 ± 2.0 nmole/ μg protein per minute; $p < 0.025$) and significant elevations in (1) postfreezing platelet Na^+/K^+ ATPase activity (6.5 ± 1.9 versus 4.3 ± 2.4 nmole/ μg of protein per minute; $p < 0.01$), (2) prefreezing and postfreezing difference in platelet Na^+/K^+ ATPase activity (5.0 ± 2.9 versus 1.2 ± 3.0 ; $p < 0.001$), and (3) frozen platelet supernatant Na^+/K^+ ATPase inhibition ($0.47\% \pm 0.4\%$ versus $0.14\% \pm 0.2\%$ inhibition of control Na^+/K^+ ATPase per microgram of protein; $p < 0.005$) and plasma Na^+/K^+ ATPase inhibition ($65\% \pm 30\%$ versus $22\% \pm 29\%$ inhibition of control Na^+/K^+ ATPase; $p < 0.001$). To evaluate the potential relationship between the freezing-induced change in platelet Na^+/K^+ ATPase activity and serum IgE, median values were calculated for the two parameters. Subjects were then

subcategorized into a 2 by 2 contingency table, and the data were analyzed by chi-square. A significant difference between subjects with low and high IgE concentrations was documented ($p < 0.01$), such that subjects with high serum IgE immunoglobulin levels tended to have large freezing-associated increases in platelet Na^+/K^+ ATPase activity.

DISCUSSION

The results of these experiments document the following in allergic subjects: (1) Na^+/K^+ ATPase activity of freshly isolated platelet membranes was reduced, (2) freezing of the platelet membranes was associated with a large increase in Na^+/K^+ ATPase activity (loss of inhibition) and the appearance of a proportional amount of Na^+/K^+ ATPase-inhibitory activity in the supernatant, and (3) plasma contained a dilutable Na^+/K^+ ATPase-inhibitory activity. A freezing-induced displacement of a Na^+/K^+ ATPase inhibitor from the platelet membrane fragments is suggested, which could result from a freezing-associated molecular rearrangement of the lipid bilayer. The inhibition was specific for the Na^+/K^+ ATPase enzyme, since the Mg^{++} and Ca^{++} ATPases were unaffected. These results support the contention that the platelet is a suitable cell type for investigating the function of the Na^+/K^+ ATPase enzyme in pathophysiologic states.¹⁷

Other investigators have suggested a relationship between abnormal metabolism or transport of cations and allergy.^{1, 2, 13, 18, 19} However, whether or not the cell fractions assayed for ATPase activity in these studies were frozen before assay was not reported. Additionally, none of these investigators reported the presence of a circulating inhibitor of Na^+/K^+ ATPase enzyme activity in humans with allergy. Interestingly, a plasma Na^+/K^+ ATPase inhibitor was reported in essential hypertension,¹⁶ a disease that, like allergic disorders, can be associated with abnormalities of smooth muscle function²⁰ and can be exacerbated both seasonally and by increased salt intake.²¹⁻²³

The same molecule was likely responsible for both the plasma and platelet membrane-inhibitory activities, since (1) incubation of allergic plasma with normal platelets reproduced the defect observed in allergic platelets and (2) the degree of plasma-inhibitory activity and the platelet membrane-enzyme activity were inversely correlated. Indeed, these results suggest that the source of the inhibitor bound to platelet membranes was the plasma. However, whether or not the same molecule was responsible for the Na^+/K^+ ATPase inhibition observed in different allergic subjects is not clear.

The present study provided no evidence to suggest

the origin or the identity of the Na⁺,K⁺ ATPase inhibitor. Potential origins include the platelet, the plasma, and another cell type. The results do support the existence of a binding site for the inhibitor on the extracellular side of the plasma membrane of the platelet. Na⁺,K⁺ ATPase inhibition was induced by the incubation of allergic plasma with intact, normal platelets. Potential extracellular binding sites include the ouabain receptor on the Na⁺,K⁺ ATPase enzyme, another enzyme site, or the lipid milieu of the enzyme. A number of endogenous, potential, natural ligands for the ouabain receptor, which are capable of inhibiting Na⁺,K⁺ ATPase activity, have been reported.²⁴⁻²⁷

The role of the inhibitor in allergic disease is open to speculation. The inhibitor could serve as a marker of the abnormal physiologic state. Alternatively, the Na⁺,K⁺ ATPase inhibitor could potentially represent a primary or secondary biochemical defect that is involved in the genesis of allergic pathophysiology. Evidence to support this hypothesis includes the broad, potential consequences of a circulating inhibitor of an enzyme that is crucial to the function of virtually all cell membranes. An altered level of responsiveness or function of a large number of cell types with a wide distribution is the hallmark of allergic disorders. These cell types include both circulating and noncirculating, tissue-bound cells.^{4-10, 20} Such a distribution is potentially compatible with a blood-borne origin for the responsible defect. Moreover, a number of the pharmacologic effects attributed to Na⁺,K⁺ ATPase inhibitors are pertinent to the described pathogenetic mechanisms of allergic disease.²⁸⁻³¹ For example, membrane depolarization can result from pump inhibition, since the Na⁺,K⁺ ATPase enzyme is electrogenic in certain cells.³² Na⁺,K⁺ ATPase inhibitors have also been reported to alter the function of both mast cells³³ and platelets,³⁴ cells that have been implicated in the pathogenesis of allergy. The postulated mechanism by which such changes in function or responsiveness could occur is by the induction of alterations of intracellular cations. Increases in intracellular Na⁺ caused by pump inhibition can result in increased intracellular Ca⁺⁺,³⁵ which could render a cell hyperexcitable. Cellular responses that depend on increases in intracellular Ca⁺⁺ include secretory activity of platelets, mast cells and basophils, aggregation of platelets, and contraction of smooth muscle.^{36, 37} All three of these processes are of cardinal importance to the pathogenesis of allergic disorders of the respiratory tract.

The clinical significance of a circulating Na⁺,K⁺ ATPase inhibitor in allergy is presently unknown. Because of the wide distribution of this integral mem-

brane protein that is critical to normal cell functioning, extensive and severe biologic effects might be the expected consequence. However, several other factors must be considered in interpreting this observation. The inhibitor likely had both its origin and primary pharmacologic action in the same tissue, possibly serving in some type of autostimulatory capacity. The inhibitor that spilled over into plasma, and was assessed in this study, may not have been in sufficient concentration to affect the functioning of other important tissues, such as the heart, which is known to be affected by cardiac glycosides. Alternatively, receptor affinities for this particular inhibitor may vary from tissue to tissue.

Likewise, the significance of a loss of 75% of normal platelet Na⁺,K⁺ ATPase activity is unknown, but a significant alteration in platelet function might be expected. Although platelet functional abnormalities have been reported in allergic subjects,⁷ a relationship to the Na⁺,K⁺ ATPase enzyme has not been established, and these impairments have not been reported to result in clinically significant bleeding disorders in allergic patients. However, several factors must be considered when an attempt is made to relate this *in vitro* observation to platelet function. First, *in vivo* physiologic compensatory mechanisms could exist and prevent widespread dysfunction of platelets. Second, platelets have a very short half-life in the circulation, providing a mechanism for the continuous replenishment of the platelet population with young, possibly unaffected, platelets. Alternatively, receptors on platelets may vary in affinity for the Na⁺,K⁺ ATPase inhibitor, resulting in subpopulations of affected cells and unaffected cells. The subpopulation of unaffected cells may then be sufficient in number to fulfill the normal physiologic functions of platelets. All of these potential explanations are based on speculation, and confirmation will await further investigation on the effects of Na⁺,K⁺ ATPase inhibition on cell function. Such studies are now underway in our laboratories with the platelet aggregation response to agonists, including adenosine diphosphate.

In summary, these data suggest that the transport-enzyme defect observed in allergic platelets was due to a circulating Na⁺,K⁺ ATPase inhibitor. *In vivo* Na⁺,K⁺ ATPase inhibition in allergy could have profound effects on intracellular cation concentrations and broad implications for the pathogenesis of allergic disease.

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