

Defect in potentiation of adenylyl cyclase correlates with bronchial hyperreactivity

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Background: Adenylyl cyclase is a transmembrane signaling system involved in the inhibition of cellular responses. Recently, we showed that the activity of adenylyl cyclase may be potentiated by stimuli that induce an increase of cellular responses but that do not activate adenylyl cyclase. This is probably an important physiologic feedback mechanism that prevents cells from becoming "overstimulated."

Objective: Because increased cellular activities are frequently observed in persons with asthma, we hypothesized that a defect in potentiation of adenylyl cyclase might be involved.

Methods: Potentiation of isoprenaline-induced adenosine cyclic monophosphate (cAMP) production with the mitogen phytohemagglutinin (PHA; 45 µg/ml) or the calcium ionophore A23187 (1 µmol/L) was studied in peripheral blood mononuclear cells taken from patients with asthma (n = 8) and healthy control subjects (n = 11).

Results: Isoprenaline-induced cAMP production was potentiated significantly in the healthy control subjects (PHA, 110% ± 15%; A23187, 92% ± 25%). In contrast, potentiation was not seen with PHA or A23187 in the total group of patients with asthma. However, some patients showed weak potentiation, whereas in others PHA decreased isoprenaline-induced cAMP production. Moreover, the effect of PHA on isoprenaline-induced cAMP production correlated significantly with the degree of bronchial hyperreactivity in patients with asthma (r = 0.96; p = 0.0001).

Conclusions: The observed defect in signal transduction could play an important part in bronchial hyperresponsiveness. (*J ALLERGY CLIN IMMUNOL* 1995;96:628-34.)

Key words: Asthma, adenylyl cyclase, bronchial hyperreactivity, potentiation

Persons with asthma show hyperreactivity of the airways to inhalation of nonspecific stimuli, such as fog, cold air, and cigarette smoke. On the basis of evidence from experimental models using mice, rats, and guinea pigs, Szentivanyi¹ hypothesized in 1968 that this bronchial hyperreactivity in patients with asthma could result from a generalized defect in the regulation of adenylyl cyclase, a now well-characterized transmembrane signaling system.² Activation of this signal-transduction system re-

Abbreviations used

cAMP:	Adenosine cyclic monophosphate
PBMC:	Peripheral blood mononuclear cells
PHA:	Phytohemagglutinin
FEV ₁ :	Forced expiratory volume in 1 second
PC ₂₀ :	Concentration causing a 20% fall in FEV ₁

sults in the production of the intracellular second messenger adenosine cyclic monophosphate (cAMP), which leads to a general dampening of cellular events (e.g., inhibition of leukocyte proliferation and interleukin production, inhibition of mediator release by leukocytes, and relaxation of smooth muscle).³⁻⁸ In previous studies at our institute in which peripheral blood mononuclear cells (PBMC) were used, it was shown that there was no defect in the activation of adenylyl cyclase in patients with asthma, as long as these patients'

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TABLE I. Clinical data of the patients

Patient No.	Sex	Age (yr)	PC ₂₀ Methacholine* (mg/ml)	FEV ₁ (%)	Inhaled β -agonists (on demand)
1	M	27	5.15	94	250 μ g terbutaline
2	M	22	0.04	100	500 μ g terbutaline
3	F	38	0.02	89	0
4	M	39	0.16	103	200 μ g salbutamol
5	F	22	2.20	83	0
6	F	37	0.25	106	250 μ g terbutaline
7	F	21	0.95	84	200 μ g salbutamol
8	M	33	2.07	100	0

*PC₂₀ methacholine determined as described in Methods.

conditions were clinically stable. Desensitization of the adenylyl cyclase system was observed only after allergen challenge, suggesting that a defect in adenylyl cyclase might be caused by the disease.^{9, 10}

Recently, however, we reported that the activity of adenylyl cyclase in PBMC may be enhanced by stimuli that do not induce adenylyl cyclase activity themselves but, to the contrary, activate transmembrane signaling systems that induce an increase of cellular activity.¹¹ This phenomenon, referred to as "potentiation," has been described in other cell types.¹²⁻¹⁴ It appears that stimuli that increase cellular activity simultaneously augment the capacity of cells to respond to cell-deactivating stimuli. Therefore potentiation of adenylyl cyclase is probably an important physiologic feedback mechanism that prevents cells from becoming "overstimulated."¹¹ In patients with asthma peripheral blood cells were shown to have a diminished response to cAMP-induced inhibition of cell proliferation and mediator release.¹⁵⁻¹⁷ Thus we hypothesized that a deficiency in the potentiation of adenylyl cyclase might be a mechanism involved in this impaired reactivity to cAMP-elevating stimuli in patients with asthma. Therefore we compared potentiation of adenylyl cyclase activity in PBMC taken from patients with asthma and healthy control subjects.

METHODS

Subjects

All patients ($n = 8$) were nonsmokers and atopic, as defined by a positive skin test result to one or more common allergens. Patients had experienced no acute asthmatic attacks for at least 2 months before the study, and there was no history of respiratory tract infections during this period. None of the patients received oral β -sympathomimetics, oral corticosteroids, or oral theophylline. All medication was stopped: inhaled corticosteroids for 4 weeks, sodium cromoglycate for 2 weeks, and inhaled β -sympathomimetics for at least 12 hours

before the study. The clinical characteristics of the patients are presented in Table I. Healthy control subjects (8 men and 3 women) were all nonsmokers and had negative skin test results and no detectable levels of IgE to common allergens. None of the control subjects received medication. Bronchial challenge with methacholine was negative for all control subjects. All subjects gave written informed consent, and the study was approved by the study center hospital medical ethics committee.

Measurement of bronchial hyperreactivity

Bronchial hyperreactivity was measured by means of a methacholine challenge test. After baseline forced expiratory volume in 1 second (FEV₁) was measured, the concentration of methacholine causing a 20% fall in FEV₁ compared with the baseline value (PC₂₀ methacholine) was measured, using a 2-minute breathing method adapted from Cockcroft et al.¹⁸ Doubling concentrations of methacholine bromide, from 0.038 mg/ml to 9.6 mg/ml, were inhaled at 5-minute intervals. Solution output was 0.13 ml/min. FEV₁ values were measured 30 and 90 seconds after each inhalation. The inhalations were stopped as soon as the fall in FEV₁ had reached 20% of the baseline FEV₁. The exact PC₂₀ was calculated from the log-dose response curve.

Measurements of cAMP production

Blood samples were taken just before the measurement of bronchial hyperreactivity. Blood was collected in tubes with ethylenediaminetetraacetic acid as anticoagulant. PBMC were isolated by means of density gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Oslo, Norway), as described by Böyum.¹⁹ Cells were washed twice in tris(hydroxymethyl)aminomethane (TRIS) buffer containing 120 mmol/L NaCl, 1 mmol/L MgCl₂, 5 mmol/L KCl, 0.6 mmol/L CaCl₂, 25 mmol/L TRIS, 5 mmol/L glucose, and 0.1% human albumin, adjusted to pH 7.4 with HCl. The final mononuclear cell fraction contained approximately 90% lymphocytes, 10% monocytes, and <1% polymorphonuclear leukocytes. Cells were suspended in TRIS buffer to a final density of 1.0 to 2.0 $\times 10^6$ cells/ml. Generation of

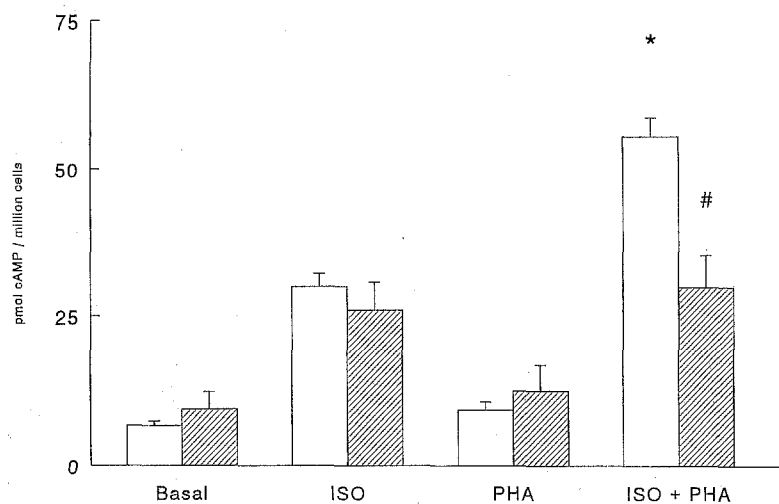


FIG. 1. Production of cAMP in PBMC from healthy controls ($n = 11$, open bars) and patients with asthma ($n = 8$, hatched bars). PBMC were stimulated for 10 minutes with isoprenaline (ISO, $1 \mu\text{mol/L}$), PHA ($45 \mu\text{g/ml}$), or isoprenaline in the presence of PHA (ISO + PHA). Results are expressed as pmol cAMP/ 10^6 cells (mean value $\pm$ SEM). (Asterisk, $p < 0.005$ ISO + PHA versus ISO [Student t test for paired observations]; number sign, $p < 0.005$ patients vs controls [Student t test for unpaired observations].)

cAMP was measured as described before.¹¹ In short, samples were preincubated with 1-methyl-3-isobutylxanthine (0.5 mmol/L final concentration) for 10 minutes. After preincubation, the samples were stimulated for 10 minutes with isoprenaline ($1 \mu\text{mol/L}$), phytohemagglutinin (PHA) ($45 \mu\text{g/ml}$), A23187 ($1 \mu\text{mol/L}$), or isoprenaline in the presence of either PHA ($45 \mu\text{g/ml}$) or A23187 ($1 \mu\text{mol/L}$). Reactions were terminated by adding 2 N HCl / 0.1 mol/L ethylenediaminetetraacetic acid, followed by boiling the samples for 5 minutes. After centrifugation of precipitated protein, the samples were neutralized with CaCO_3 and cAMP was determined by using the cAMP binding protein assay as described by Meurs.²⁰ Results are expressed as pmol cAMP/ 10^6 cells. Experiments with PHA were performed on PBMC taken from 11 healthy control subjects and eight patients with asthma, except for dose-response curves of isoprenaline, which were done for three healthy control subjects and five patients with asthma. Experiments with A23187 were done with four healthy control subjects and four patients with asthma.

Materials

Lymphoprep was obtained from Nycomed Pharma, Oslo Norway. L-isoproterenol, A23187, and 1-methyl-3-isobutylxanthine were obtained from Sigma Chemical Co., St. Louis, Mo. PHA was obtained from Wellcome Diagnostics, Dartford, England. cAMP kits were from the Radiochemical Centre, Amersham U.K.

Statistical analysis

Statistical analysis was performed with the SPSS/PC+ Statistical Package (SPSS Inc., Chicago, Ill.). The Student t test for paired and unpaired observations was used for statistical analysis of the results of group means; for

regression analysis, the Pearson correlation coefficient was calculated. Values refer to the mean $\pm$ SEM.

RESULTS

PBMC were stimulated with the β -adrenergic stimulus isoprenaline ($1 \mu\text{mol/L}$) in the absence or presence of the lectin PHA, a common leukocyte activator (PHA, 45 mg/ml). The production of cAMP induced by isoprenaline was found to be identical in PBMC taken from healthy control subjects ($n = 11$) and patients with asthma ($n = 8$) (Fig. 1). Isoprenaline-induced cAMP production in PBMC from healthy control subjects could be potentiated significantly by incubating the cells simultaneously with PHA, whereas PHA alone had no significant effect on cAMP production. (The small cAMP production induced by PHA is probably due to endogenous production of histamine induced by PHA²¹). In contrast, the potentiating effect of PHA was absent in PBMC taken from patients with asthma (Fig. 1). As was reported previously, potentiation of adenylyl cyclase activity in PBMC from healthy controls ($n = 3$) was not dependent on the concentration of isoprenaline.¹¹ Likewise, the lack of potentiation of adenylyl cyclase activity in PBMC from patients with asthma ($n = 5$) proved to be independent of the concentration of isoprenaline (Fig. 2).

Potentiation of isoprenaline-induced cAMP production in PBMC from healthy controls was not restricted to PHA but could also be induced by

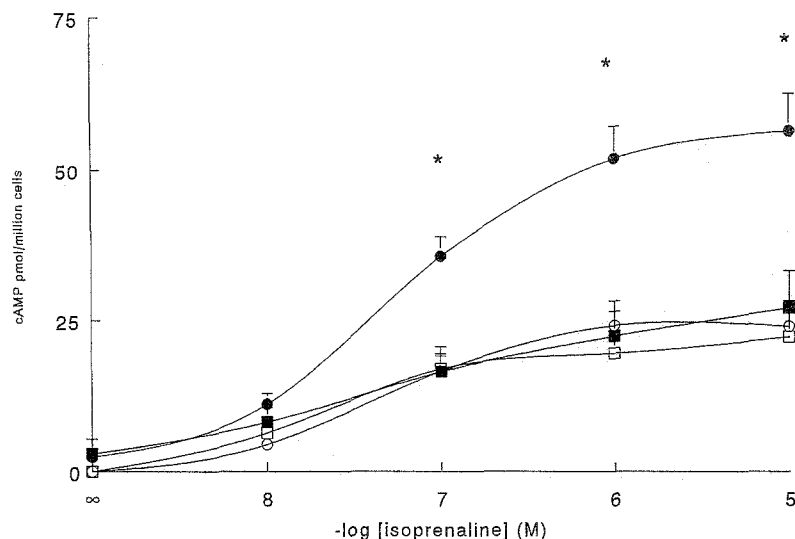


FIG. 2. Production of cAMP in PBMC from healthy controls ($n = 3$, circles) and patients with asthma ($n = 5$, squares). PBMC were stimulated either with increasing concentrations of isoprenaline in the absence (*open symbols*) or presence (*closed symbols*) of PHA (45 $\mu\text{g/ml}$). Experimental conditions were as in Fig. 1. Results are expressed as cAMP production above basal value (mean value $\pm$ SEM). (Asterisk, $p < 0.05$ controls vs patients [Student t test for unpaired observations].)

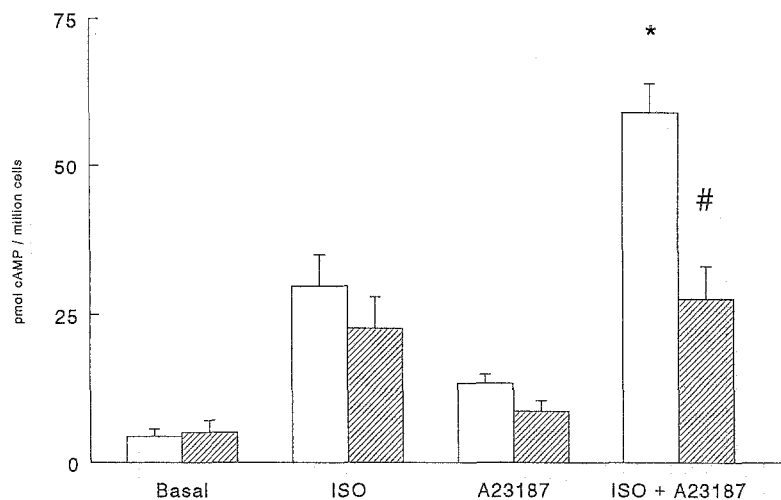


FIG. 3. Production of cAMP in PBMC from healthy controls ($n = 4$, open bars) and patients with asthma ($n = 4$, hatched bars). PBMC were stimulated with isoprenaline (1 $\mu\text{mol/L}$), A23187 (1 $\mu\text{mol/L}$), or isoprenaline in the presence of A23187. Experimental conditions were as in Fig. 1. Results are expressed as pmol cAMP/ 10^6 cells (mean value $\pm$ SEM). (Asterisk, $p < 0.05$ ISO + A23187 vs ISO [Student t test for paired observations]; number symbol, $p < 0.05$ patients vs controls [Student t test for unpaired observations].)

another leukocyte activator, the calcium ionophore A23187¹¹ ($n = 4$). As was the case for PHA, in PBMC from patients with asthma ($n = 4$) A23187 did not induce potentiation of cAMP production (Fig. 3). So potentiation of adenylyl cyclase was deficient in PBMC from patients with asthma for both leukocyte activators tested.

Although we did not observe significant potentiation of adenylyl cyclase in the total group of patients, a small amount of potentiation was found in PBMC from some patients, whereas in other patients adenylyl cyclase activity was even decreased by co-stimulation with PHA. Because the patients also showed some variation in the degree

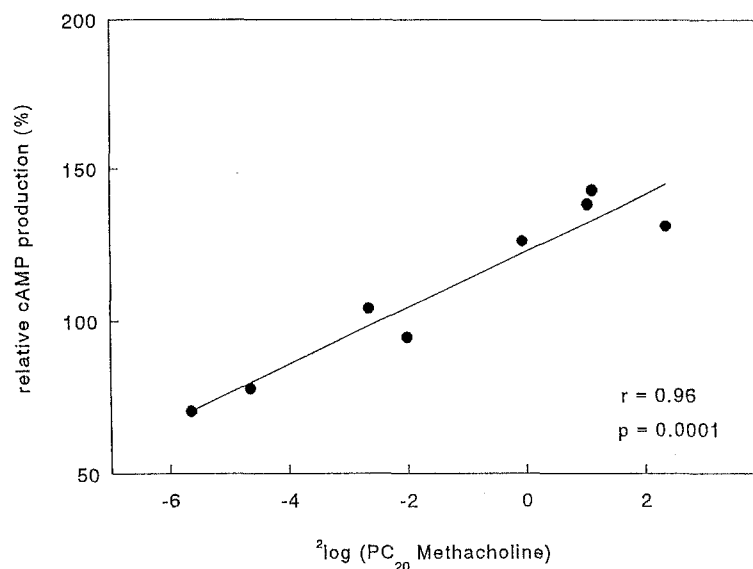


FIG. 4. Correlation between potentiation of cAMP production in PBMC from patients with asthma ($n = 8$) and bronchial hyperresponsiveness. PBMC were stimulated for 10 minutes with isoprenaline ($1 \mu\text{mol/L}$), PHA ($45 \mu\text{g/ml}$), or isoprenaline in the presence of PHA. The potentiation, expressed as the relative cAMP production, was calculated for individual patients according to the following formula:

$$\text{Relative cAMP production (\%)} = \frac{\text{cAMP production (ISO + PHA)}}{\text{cAMP production (ISO)}} \times 100\%$$

cAMP production (ISO) was corrected for basal cAMP production, and cAMP production (ISO + PHA) was corrected for cAMP production (PHA) to compensate for additive effects of PHA.

of their bronchial hyperresponsiveness (Table I), we evaluated whether these two parameters were in some way related. There proved to be a significant correlation between the residual amount of potentiation in PBMC (expressed as relative cAMP production) and the bronchial hyperresponsiveness in these patients ($n = 8$) (Fig. 4). The patients whose PBMC were the least hyperreactive did show a small amount of potentiation of adenylyl cyclase activity (in control subjects the relative potentiation was $210\% \pm 15\%$), whereas in patients who were more hyperreactive, adenylyl cyclase activity was even decreased by PHA.

DISCUSSION

This study shows that cAMP production induced by β -adrenergic receptor stimulation alone is not deficient in PBMC taken from patients with asthma compared with healthy control subjects, as was predicted by Szentivanyi's theory¹ (Figs. 1-3). This is in agreement with our previous studies in PBMC membranes that showed that in patients with asthma who are clinically stable a desensitization of the adenylyl cyclase system is observed only after allergen challenge.^{9, 10} However, in this study

we demonstrated that, although being not deficient in cAMP production when stimulated only with a β -adrenergic stimulus, PBMC from patients with asthma who are clinically stable lack the capacity to potentiate adenylyl cyclase activity when co-stimulated with the mitogens PHA or A23187, whereas in PBMC from healthy control subjects adenylyl cyclase activity was potentiated significantly under these conditions.

Activation of adenylyl cyclase is important in the inhibition of cellular activities,³⁻⁸ and as was shown previously,¹¹ potentiation of adenylyl cyclase by cell-activating stimuli is probably a biochemical feedback mechanism to prevent cells from becoming overly stimulated. Therefore the observed lack of potentiation in PBMC from patients with asthma would imply that these cells are less resistant to activating stimuli. In fact, in patients with asthma an impairment of the inhibitory effect of cAMP-elevating agents was found on the proliferation of PBMC,¹⁶ on the inhibition of respiratory burst,¹⁵ and on lysosomal enzyme release in granulocytes.¹⁷ The observed lack of potentiation of adenylyl cyclase could provide a molecular explanation for these findings.

The biomolecular mechanism underlying the loss of potentiation in PBMC from patients with asthma is not yet fully understood. In a previous report we showed that in PBMC potentiation of adenylyl cyclase is mediated by the activation of the calcium-dependent enzyme calmodulin as a result of hydrolysis of phosphatidyl-inositol-bisphosphate and subsequent increase in intracellular calcium.¹¹ According to this model, the lack of potentiation in PBMC from patients with asthma could be due to a defect somewhere in this cascade of biochemical events. Recently, it was shown by Meurs et al.²² that there was no difference in the production of inositol-phosphates in PBMC between patients with asthma and healthy control subjects.²² Therefore it may be concluded that the defect in the mechanism of potentiation of adenylyl cyclase in PBMC from patients with asthma is located distal from this first step in the described pathway.

PBMC are a mixture of various cell types. Therefore differences in the cell type distribution between control subjects and patients with asthma could play a role in the observed lack of potentiation in PBMC from persons with asthma. However, several studies reported neither differences between control subjects and persons with asthma in differential leukocyte counts in peripheral blood,^{23, 24} nor differences in the expression of activation markers on T cells (CD4, CD8, CD25, and HLA-DR).²⁵ Therefore differences in cell type distribution are not likely to explain the observed lack of potentiation of adenylyl cyclase activity in PBMC from patients with asthma.

A strong correlation was found between the degree of hyperreactivity in the patients and the small residual amount of potentiation of adenylyl cyclase in PBMC (Fig. 4). This correlation suggests a relationship between the observed defect in signal transduction in PBMC and this clinical parameter of asthma. Two mechanisms could be involved. First, as a modification of the original theory of Szentivanyi, the lack of potentiation of cAMP production could be a generalized defect and would therefore also be present in bronchial smooth muscle, in which cAMP is known to induce relaxation.⁸ An impairment in the capacity of potentiation of cAMP production in the presence of a bronchoconstrictive stimulus (e.g., methacholine) could result in a disturbed balance between contraction and relaxation of bronchial smooth muscle. That the lack of potentiation in patients with asthma is not necessarily restricted to PBMC is suggested by the impaired inhibitory responses

to cAMP-elevating agents reported in granulocytes.^{15, 17} Second, because adenylyl cyclase is important in the regulation of cytokine production in mononuclear cells,^{5, 6, 26-28} a lack of potentiation of adenylyl cyclase could result in altered cytokine production that would initiate a chronic inflammatory response in lung tissue indirectly, causing an enhanced bronchial responsiveness. Altered cytokine production was recently reported in patients with asthma both in vivo and in vitro.²⁹⁻³¹

In conclusion, in this study we have presented data that revitalize the theory that a defect in adenylyl cyclase activity is a mechanism in bronchial asthma, as was formulated by Szentivanyi more than two decades ago, albeit at a more complicated level than originally proposed.

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