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Increased levels of protein kinase C in lymphocytes in asthma: possible mechanism of regulation

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ABSTRACT: Asthma is an inflammatory disease of the airways. Activation of lymphocytes leads to elaboration of inflammatory mediators which are likely to initiate and perpetuate the asthmatic response. During activation of lymphocytes, the role of protein kinase C (PKC) has been emphasized. Therefore, changes in PKC activity in peripheral blood lymphocytes in bronchial asthma can be expected, which may be due to alterations in the regulatory mechanisms of the enzyme molecule. To understand the mechanism of regulation of PKC activity the effects of drugs, such as carbachol, histamine, sphingosine and disodium cromoglycate (DSCG), on the lymphocytes of healthy subjects were studied.

The study included 27 asthmatic patients and 14 healthy volunteers. Disease was classified as mild, moderate-to-severe, and cases in remission. PKC activity was determined in peripheral blood lymphocytes by [^{3}H] phorbol 12,13-dibutyrate ([^{3}H]PDBu)-binding assay.

A highly significant ($p<0.001$) increase was observed in total, cytosolic and membrane PKC activity in all of the asthmatic patients as compared to the healthy group. There was an increased translocation of PKC from cytosol to membrane in all of the groups, and the extent of translocation of the enzyme indicates physiological activation of the cells. A highly significant ($p<0.001$) reciprocal relationship ($r=-0.47$) existed between forced expiratory volume in one second (FEV_1) as percentage predicted and total PKC activity in bronchial asthma. Carbachol and histamine significantly ($p<0.001$) increased PKC activity in lymphocytes, the increase being dose-dependent for histamine. Sphingosine or disodium cromoglycate (DSCG) brought about complete inhibition of PKC activity at 100 nM.

We conclude that protein kinase C activity is increased in lymphocytes in bronchial asthma. Our findings suggest that the mediators (carbachol and histamine), and drugs (sphingosine and disodium cromoglycate) possibly exert their action on protein kinase C by influencing the regulatory domain of the enzyme.
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Inflammation of the airways is an important determinant of hyperreactivity in the pathogenesis of asthma [1, 2]. Bronchial biopsies and bronchial lavage fluid obtained from patients with asthma show an infiltration of inflammatory cells, such as lymphocytes, macrophages, mast cells, eosinophils, *etc*. [3, 4]. Lymphocytes are now considered to make a significant contribution to the pathophysiology of asthma. It has been suggested that, fundamentally asthma may be mediated by a difference in the type of lymphocytes, predominantly in the airway mucosa. This may be due to sensitization of a subpopulation of CD4+ T-lymphocytes, particularly the type 2 T-helper (Th2) subtype [1]. In the activated state, these cells secrete a limited number of cytokines, including interleukin (IL)-4 and IL-5, which have the capacity to develop the asthmatic phenotype and differentiate B-cells, with the capacity to produce immunoglobulin E (IgE), leading an initiation and perpetuation of the asthmatic response [5].

During lymphocyte activation, stimulation of T-cell antigen receptor-induced activation of phospholipase C (PLC) occurs, which hydrolyses the phosphatidylinositol 4,5-diphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and sn 1,2-diacylglycerol (DAG), both of which lead ultimately to the activation of protein kinase C (PKC). Therefore, the pivotal role of PKC in T-cell activation has been emphasized [6]. With the current theories suggesting the role of T-cells and their products in the persistent inflammation in airways in asthma [7], and an important role attributed to PKC in activation of lymphocytes, it might be envisaged that changes in PKC activity may take place in lymphocytes in bronchial asthma. In the present investigation, we attempted to determine the status of PKC activity in the lymphocytes in bronchial asthma and to correlate it with the severity of the disease.

Studies on the mechanism of regulation of PKC may be helpful in understanding the mode of activation of lymphocytes in asthma. Since the biochemical characteristics of any particular enzyme, its make-up and mechanism of regulation in a particular cell type in a species are fundamentally similar, experiments elucidating the

mechanism of regulation of PKC in the lymphocytes of healthy subjects, using asthmogenic stimuli (carbachol and histamine), a potent inhibitor of PKC (sphingosine) and an anti-inflammatory drug (disodium cromoglycate (DSCG)), were conducted, as presented in this manuscript.

Materials and methods

Subjects

The study included 27 patients with bronchial asthma (mean±SD age 32±11 yrs, range 17–65 yrs), and 14 normal healthy subjects (mean±SD 25±2 yrs, range 21–29 yrs) of either sex. General and systemic examinations were performed. This was followed by spirometric evaluation (Morgan Transfer Test Model C; P.K. Morgan Ltd., Kent, UK) of patients. Forced vital capacity (FVC), forced expiratory volume in one second (FEV_1 % predicted), forced respiratory capacity (FRC), respiratory volume (RV) and total lung capacity (TLC) were measured. Asthmatic patients were classified into groups as: mild (FEV_1 60–80% pred); moderate-to-severe (FEV_1 <60% pred); and cases in remission (FEV_1 >80% pred). Detailed disease history for each patient, including age, sex, smoker (cigarettes·day^{-1} × yrs), severity (*i.e.* FEV_1 % pred), and medication were recorded (table 1). Bronchodilators, if used, were withdrawn 1 day before testing to establish baseline lung function and collection of blood samples. Twenty one patients were receiving regular inhaled beclomethasone dipropionate (800–1,600 µg), and one patient was receiving oral prednisolone. These drugs were allowed to be continued.

Reagents

[^{3}H] phorbol 12, 13-dibutyrate ([^{3}H] PDBu; specific activity 23.5 Ci·mM^{-1}) was purchased from Amersham International (Amersham, UK). Bovine serum albumin (BSA), carbachol, dithiothreitol (DTT), ethylenediamine tetra-acetic acid (EDTA), 2-ethylene glycol bis (β-aminoethyl-ether) N,N,N',N' tetra-acetic (EGTA), histamine, phenylmethyl sulphonyl fluoride (PMSF), phosphatidylserine (PS), sphingosine, tris (hydroxymethyl) aminomethane (Tris-HCl) and trypan blue were purchased from Sigma Chemical Co. (St. Louis, USA). Lymphoprep (specific gravity 1.077) was purchased from Nyegaard Co., (A/S Oslo, Norway). Disodium cromoglycate (DSCG) was a generous gift from M/s Rallis India Ltd (Ankhleshwar, Gujrat, India). Heparin (5,000 IU·mL^{-1}) was obtained from Biological Evans Ltd (Hyderabad, India). All other chemicals used were of analytical grade.

Table 1. – History of disease

Pt No.	Age yrs	Sex	Smoker	FEV_1 % pred	Medication
Asthma in remission					
1	40	M	No	102	Inh BDP + Sal, Tab Theo
2	24	M	No	86	Tab Theo + Sal
3	22	M	No	81	Inh BDP + Sal
4	26	F	No	107	Inh BDP + Sal, Tab Theo
5	17	F	No	92	Tab Theo + Sal
6	22	F	No	96	Inh BDP + Sal
7	27	M	No	94	Inh BDP + Sal, Tab Theo
8	24	M	No	92	Inh BDP + Sal, Tab Theo + Sal
9	30	M	Yes#	90	Inh BDP + Sal, Tab Theo + Sal
10	26	M	No	88	Inh BDP + Sal, Tab Terf
11	31	M	No	89	Inh BDP + Sal, Tab Theo
12	30	M	No	87	Inh BDP + Sal, Tab Theo
13	65	M	No	93	Tab Theo + Sal
14	27	M	No	81	Tab Theo + Sal
Mild asthma					
15	22	F	No	67	Inh BDP + Sal
16	19	M	No	68	Inh BDP, Tab Theo
17	35	M	No	80	Inh BDP
18	25	M	No	77	Tab Theo + Sal
19	26	M	No	71	Inh BDP + Sal, Tab Theo
20	28	F	No	72	Tab Theo + Sal
21	29	M	No	71	Inh BDP + Sal
Moderate-to-severe asthma					
22	45	M	No	30	Inh BDP, Tab Pred, Tab Sal
23	55	M	No	52	Inh BDP
24	45	M	No	55	Inh BDP, Inh cromolyn
25	36	M	No	49	Inh BDP, Tab Sal
26	42	F	No	49	Inh BDP + Sal, Tab Theo + Sal
27	34	M	Yes‡	27	Inh BDP + Sal, Tab Theo + Sal

#: 2 cigarettes·day^{-1}×10 yrs; ‡: 10 cigarettes·day^{-1}×15 yrs; Pt: patient; M: male; F: female; FEV_1: forced expiratory volume in one second; % pred: percentage of predicted value. Inh: inhaler; Tab: tablet; Theo: theophylline; BDP: beclomethasone dipropionate; Sal: salbutamol; Terf: terfenidine; Pred: prednisolone.

Preparation of peripheral blood lymphocytes

Peripheral blood lymphocytes were prepared by the method of BOYUM [8]. Briefly, 10 mL of blood was collected by venipuncture in heparinized (30 IU heparin·mL^{-1} blood) sterile stoppered vials. Blood was diluted 1:1 with physiological saline (0.15 M NaCl), carefully layered on 10 mL of lymphoprep and centrifuged at 1,500 rpm for 10 min. The interface containing lymphocytes was collected, diluted with physiological saline and centrifuged in the same way. Contaminating erythrocytes were removed by washing the pellet with 0.85% ammonium chloride solution. The pellet was then washed twice with physiological saline, and finally suspended in it. The viability of cells was checked by trypan blue exclusion, which was above 97% in all sets of experiments. Total cell counts were performed and concentration adjusted to give 40×10^6 cells·mL^{-1}. The differential count after Giemsa staining revealed 89±2% lymphocytes.

Preparation of cell lysate

A cell suspension (40×10^6 cells·mL^{-1}) in sonication buffer (25 mM Tris HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF and 250 mM sucrose) was sonicated in an ultrasonic processor (Model W-385, Farmingdale, NY, USA; Heat system-Ultrasonic Inc.) using a microtip, by giving four bursts of 30 s at 1 min intervals. During sonication, the cell suspension was immersed in an ice bath. Lysis of cells was checked under a light microscope.

Subcellular fractionation

Sonicated cell lysate was centrifuged at 105,000×g for 1 h at 4°C in a Beckman L 7-65 (Beckman Instrument Inc., CA, USA) ultracentrifuge. The supernatant (cytosol) was saved and the pellet (membrane) suspended in 0.6% triton X-100 in sonication buffer. The suspension was allowed to stand at 4°C for 2 h for dissolution of membranes. Protein in cytosol and membrane fractions was determined by the method of LOWRY *et al*. [9].

Protein kinase C (PKC) assay

PKC was assayed by the [^{3}H] PDBu-binding method as described previously [10]. Briefly, the reaction system (50 µL) consisting of 50 mM Tris HCl (pH 7.5), 500 µM $CaCl_2$, 2 mM DTT, 0.1% BSA, 5 µg PS, [^{3}H] PDBu (50,000–60,000 DPM) and 30–60 µg protein (enzyme), was incubated at 30°C for 25 min. The reaction was terminated by adding 2 mL of chilled 10 mM Tris HCl (pH 7.5). Contents were filtered on Whatman glass fibre filters (GF/C) and washed twice with 10 mM Tris HCl (pH 7.5). Filters were processed for radioactivity counting in a liquid scintillation counter (Beckman LS 6000 IC). Nonspecific binding was measured in the presence of 5 µM phorbol myristate acetate (PMA) and subtracted from the total binding of [^{3}H] PDBu to obtain the PKC activity, which was expressed as femtomoles of [^{3}H] PDBu bound to protein present in one million lymphocytes under the experimental conditions.

Data analysis

Values are expressed as mean±SD. Student's t-test was applied for statistical analysis. A p-value less than 0.05 was considered significant.

Results

Total PKC activity and its distribution in cytosol and membrane fractions of lymphocytes in controls and various asthmatic groups is presented in figure 1. In controls, the total activity was observed to be 2.8±0.6 (range 2.0–4.1 femtomoles bound [^{3}H] PDBu·10^{-6} lymphocytes); in mild asthmatics, it was 14.0±9.1 (range 6.2–30.5 fmoles); in moderate-to-severe cases it was 19.2±8.9 (range 13.1–33.5 fmoles); and in cases in remission, it was 9.8±3.1 (range 6.9–18.5 fmoles). There was a significant ($p<0.001$) increase in total, cytosolic and membrane PKC activity in all asthmatic patients in comparison with the control group. Of the total PKC activity in the control group, 80% was present in cytosol fraction and the remaining in membrane fraction, while in asthmatic groups, of the total PKC activity, 71%, 73% and 69% was present in cytosolic fractions in mild, moderate-to-severe asthmatics and in cases in remission, respectively. These data suggest an increase in the expression of PKC activity, associated with an increased translocation of more than 9% from cytosol to membrane in all asthmatics compared to control group. Furthermore, there was a significant ($p<0.001$) reciprocal correlation ($r=-0.47$) between FEV1 % predicted and total PKC activity of asthmatic patients (fig. 2).

To understand the regulatory mechanism of PKC, the effect of drugs on lymphocytes obtained from healthy volunteers was studied. Treatment of lymphocytes with carbachol and histamine caused a significant ($p<0.001$) increase in total PKC activity at all the concentrations used. Carbachol increased PKC activity linearly up to

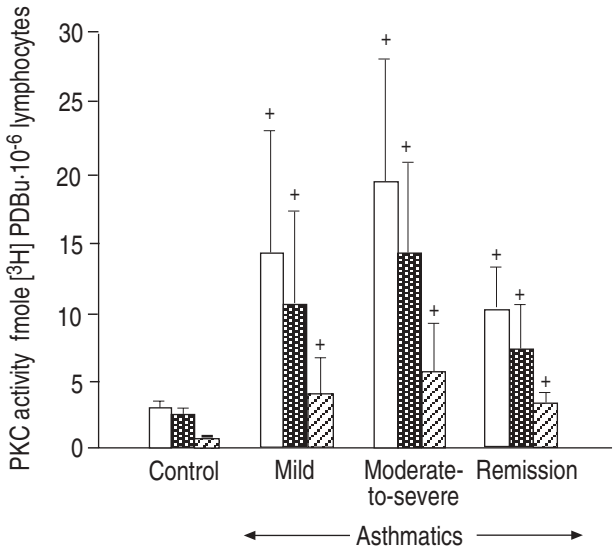

Fig. 1. – PKC activity in lymphocytes of asthmatics and controls. Values are expressed as mean±SD femtomoles bound [^{3}H] PDBu·10^{-6} lymphocytes. $^+$: $p<0.001$, as compared to control. ▭ : total; ▩ : cytosol; ▨ : membrane. PKC: protein kinase C; [^{3}H] PDBu: [^{3}H] phorbol 12–13-dibutyrate.

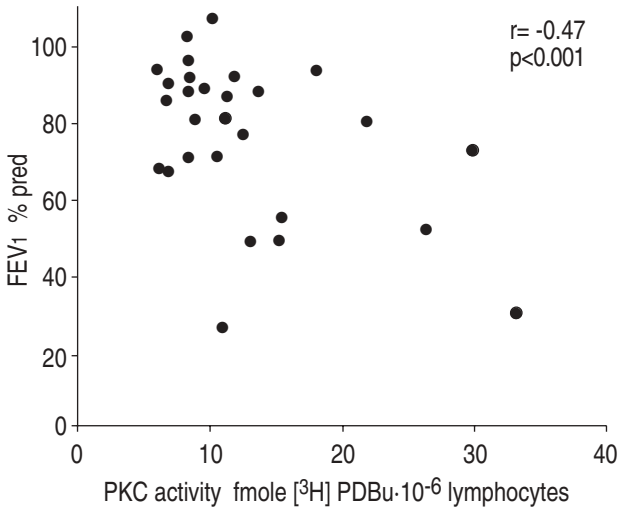

Fig. 2. – Relationship between FEV1 % predicted and PKC activity expressed as femtomoles bound [^{3}H] PDBu·10^{-6} lymphocytes of asthmatic patients. FEV1: forced expiratory volume in one second. For further definitions see legend to figure 1.

100 nM concentration, followed by a reduced level of stimulation at 1,000 nM, attaining an intermediate value between 1 and 10 nM (fig. 3). Histamine caused a dose-related increase up to 1,000 nM. A similar significant ($p<0.001$) increase in cytosolic and membrane PKC activity was observed in the presence of carbachol or histamine. Physiological activation of lymphocytes has been reported to occur at 5% translocation of PKC from cytosol to membrane [11]. In the present study, the distribution pattern showed that translocation of PKC from cytosol to membrane increased by 12% in carbachol-

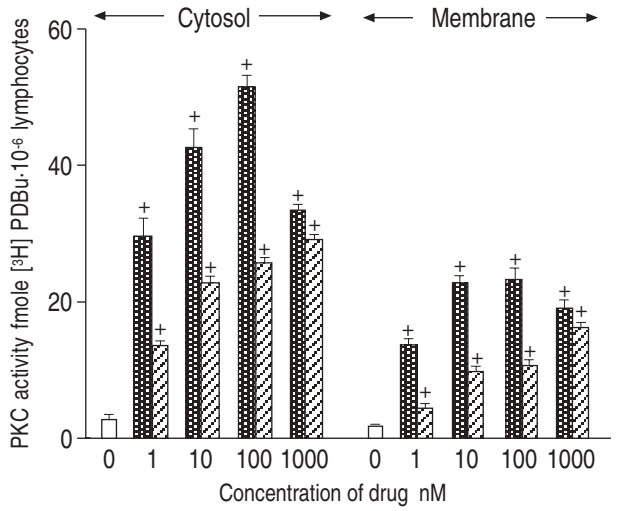

Fig. 3. – Effect of carbachol and histamine on distribution of PKC activity in cytosol and membrane fractions of lymphocytes of healthy subjects (expressed as femtomoles bound [^{3}H] PDBu·10^{-6} lymphocytes (mean±SD of three different subjects)). Experimental conditions: in a total volume of 500 µL, in Hanks' balanced salt solution. 6×10^6 cells were incubated in various concentrations of carbachol and histamine for 10 min and then centrifuged at 6,000 rpm for 2 min in an Eppendorf centrifuge. The cell pellet was washed twice with physiological saline and finally suspended in 150 µL of sonication buffer. Cytosol and membrane fractions were prepared as reported in "Materials and methods". PKC activity is expressed as femtomoles bound [^{3}H] PDBu·10^{-6} lymphocytes. +: $p<0.001$, as compared to control. ▭ : control; ▩ : carbachol; ▨ : histamine. For definitions see legend to figure 1.

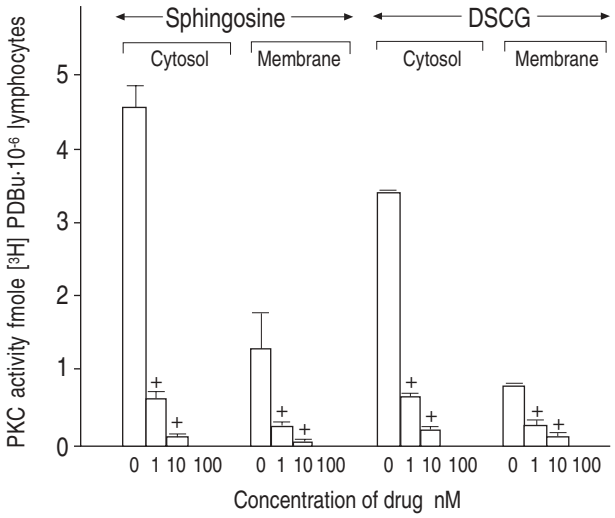

Fig. 4. – Dose response of sphingosine and disodium cromoglycate (DSCG) on PKC activity in cytosol and membrane fractions of lymphocytes of healthy subjects (expressed as femtomoles bound [^{3}H] PDBu·10^{-6} lymphocytes (mean±SD of three different subjects)). For experimental conditions see legend to figure 3. For definitions see legend to figure 1. +: $p<0.001$, as compared to control.

treated cells at 1 nM and above. In histamine-treated cells, translocation was dose-dependent, but more than 5% (*i.e.* 10%) was achieved at 10 nM concentration and above (fig. 3).

The effect of sphingosine and DSCG was studied in a similar fashion to that of carbachol and histamine. Sphingosine or DSCG markedly inhibited PKC activity, and more than 90% inhibition was achieved at 10 nM concentration (fig. 4). PKC activity was completely inhibited at 100 nM concentration of either of the two drugs. There was a highly significant ($p<0.001$) inhibition of cytosolic and membrane PKC activity at all the concentrations of sphingosine or DSCG used (fig. 4).

Discussion

T-lymphocytes have been considered to play a central role in the inflammation occurring in bronchial asthma [7]. The increased expression of activation markers on T-lymphocytes suggests that T-cell activation plays a role in the pathogenesis of bronchial asthma [12, 13]. Therefore, changes in protein kinase C in lymphocytes in bronchial asthma were examined.

PKC is a serine/threonine phosphotransferase enzyme involved in several biochemical events in the cell [14]. It has been reported to participate in inflammation, immune response and several other physiological functions [15, 16]. In the present study, a significant elevation in PKC activity was observed in the lymphocytes of all asthmatic patients as compared to the healthy subjects. The rise was 4.9 fold in mild, 6.8 fold in moderate-to-severe and 3.5 fold in cases of asthma in remission. The increased levels of PKC in mild and moderate-to-severe cases indicate activation of lymphocytes, which tend to decline in remission, suggesting that the disease severity is associated with the variation in the T-lymphocytes [17].

PKC is predominantly cytosolic in most tissues, where it remains in inactive form. Its translocation to

the membrane converts it to the fully active form [14, 18]. As little as 5% translocation of PKC from cytosol to membrane has been reported to be adequate for biological activity of lymphocytes [11]. In the present investigation, more than 9% translocation of enzyme from cytosol to membrane was observed in all groups of asthmatic patients, suggesting a physiological activation of lymphocytes in the disease. The activation of PKC may be due to the stimulation of T-cell antigen receptors, which induces phopholipase C (PLC) activity in lymphocytes. PLC in turn hydrolyses phosphatidylinositol 4,5-diphosphate (PIP_2) yielding inositol 1,4,5-triphosphate (IP_3) and sn 1,2-diacylglycerol (DAG), the two second messengers [6]. These second messengers are known to bring about activation of PKC [14, 16]. PKC may also be activated by lysophosphatidylcholine (LPC) and other cis-unsaturated fatty acids produced by the action of phospholipase A_2 on phosphatidylcholine or DAG [15].

Increased activity of PLA_2 and LPC levels has been reported in lymphocytes of asthmatic patients [19], which may potentiate PKC activity, as observed in this investigation. Production of DAG and arachidonic acid have also been reported in lymphocytes of asthmatic patients [20]. However, there is no statistical difference in the concentrations of these two compounds between asthmatic subjects and healthy individuals. This may be due to the rapid metabolism of these compounds, where DAG may liberate free cis-unsaturated fatty acids that may bring about activation of PKC [15], and arachidonic acid may be converted to products of the lipoxygenase pathway, which are known mediators of inflammation and asthma [16, 21]. The lipoxygenase metabolites are also known to activate PKC through DAG during Hela cell attachment and spreading [22]. A similar situation may exist for PKC activation in lymphocytes in bronchial asthma as observed in this investigation. Once PKC is activated, there is activation of lymphocytes. The activated T-lymphocytes of CD4+ phenotype release several important cytokines, such as IL-4 and IL-5, which influence the differentiation and function of eosinophils and mast cells that lead to epithelial damage, inflammation in the airways and the pathogenesis of asthma [5, 23, 24].

In order to assess the regulation of PKC activity, the effect of various drugs on lymphocytes of healthy subjects was studied. The molecular structure of PKC has been reported to have two domains, *i.e.* catalytic and regulatory [14, 25]. The activity of the enzyme is regulated by changes in either of the two domains caused by binding of the appropriate molecule(s) to them. The regulatory domain has a binding site for DAG, which is the physiological modulator of the enzyme [14]. Carbachol may influence PKC activity through formation of DAG from hydrolysis of polyphosphoinositides by the action of PLC after stimulation of cholinergic receptors on the activated human lymphocytes [26]; thus, acting as an agonist of PKC by influencing the action at its regulatory domain. Similarly, sphingosine, a known antagonist of PKC, acts by binding to the regulatory domain of the PKC. Sphingosine can be delivered into the cell due to its ampiphilic nature, and is assumed to become partitioned in the membrane. It competitively inhibits the binding of DAG on the regulatory domain of PKC [27]. Thus carbachol and sphingosine change PKC activity by influencing the regulatory domain of the enzyme. In the present study, carbachol, at a concentration of 100 nM, was able to activate the enzyme activity by 25 fold. A similar dose-dependent activation was caused by histamine, but the linearity could be observed only in the concentration range of 1–10 nM. This is apparently within the physiological range of histamine, which is reported to be 5–10 nM in plasma in 15–20 min after challenge with an allergen [28].

The decline in activation of PKC activity at a 1,000 nM concentration of carbachol may be due to downregulation of the enzyme, similar to the downregulation caused by a high concentration of PMA, which is a well-known activator of PKC [29]. However, such a change with histamine could not be observed in the present investigation, possibly because the concentration used was not sufficient to saturate the histamine receptors on lymphocytes, the number and subtypes of which have been shown to change in bronchial asthma [30]. These findings suggest that histamine, at physiological concentrations, activates PKC in a manner similar to carbachol, presumably by increasing the DAG formation and its subsequent binding to the regulatory domain of the enzyme. This was further confirmed by studying the effect of sphingosine, which caused about 90% inhibition of PKC activity at 10 nM and absolute inhibition at 100 nM concentration. A similar pattern was observed when lymphocytes were incubated with DSCG, suggesting that the drug inhibits PKC activity, possibly by acting at the regulatory domain of the enzyme, thereby supporting the contention of SAGI-EISENBERG [31].

PKC represents a group of 12 isoforms [16]. With the exception of PKCγ all have been detected in T-lymphocytes [6]. PKCβ is the dominant form expressed in T-cells, suggesting its preferential role during the activation of T-cells [32, 33]. Determining the isoenzymes of PKC may indicate its precise role in asthma, which has not so far been achieved. However, the finding that PKC activity is appreciably increased, strongly suggests the stimulus-induced activation of a PKC-mediated signal transduction pathway during the activation of lymphocytes and that it has a role in the pathophysiology of asthma. The PKC activity is regulated by various drugs, possibly by their influence on the regulatory domain of the enzyme.

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