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Calreticulin transacetylase (CRTAase): Identification of novel substrates and CRTAase-mediated modification of protein kinase C (PKC) activity in lymphocytes of asthmatic patients by polyphenolic acetates*

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Abstract: Earlier reports from our laboratory established the acetyl transferase function of calreticulin (CRT), enabling CRT to transfer acetyl groups from the acetoxy groups of polyphenolic acetates (PAs) to certain receptor proteins. We have in this paper documented the ability of CRT to catalyze the possible transfer of acetyl moiety from 7-acetamido-4-methylcoumarin (7-N-AMC) to the proteins, glutathione S-transferase (GST), and NADPH cytochrome c reductase, leading to the modification of their catalytic activities. 7-Acetoxy-4-methylthiocoumarin (7-AMTC) compared to 7-acetoxy-4-methylcoumarin (7-AMC) when used as a substrate for calreticulin transacetylase (CRTAase) yielded significantly higher catalytic activity. PM3-optimized geometries suggested that the availability of electrons on the sulfur atom of the thiocarbonyl group of the thiocoumarin may render the substrate binding more favorable to the active site of the enzyme as compared to its oxygen analog. Further CRTAase activity was characterized in the human blood lymphocytes. There was no appreciable difference in CRTAase activity of lymphocytes of asthmatic patients as compared to those of normal subjects. The results presented here highlight for the first time the irreversible inhibition of human blood lymphocytes protein kinase C (PKC) by 7,8-diacetoxy-4-methylcoumarin (DAMC) possibly by way of acetylation. The activity of PKC in lymphocytes of asthmatic patients was found to proportionally increase with the severity of the disease. When PA was incubated with lymphocytes of normal patients, PKC was inhibited marginally. On the other hand, lymphocyte PKC of severe asthmatic patients was inhibited drastically. Several PAs inhibited PKC of asthmatic patients in tune with their specificity to CRTAase. DAMC was found to exert maximum inhibitory action on PKC, while

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7,8-dihydroxy-4-methylcoumarin (DHMC), the deacetylated product of DAMC, failed to inhibit PKC. These observations clearly describe DAMC as the novel irreversible inhibitor of PKC, and DAMC may be found useful in the control of inflammation and may serve as a potential drug candidate in the therapy of asthma.

Keywords: polyphenolic acetates; calreticulin; transacetylase; nitric oxide synthase; asthma; protein kinase c.

INTRODUCTION

There is a wealth of information on the diverse biological activities of polyphenols [1], such as coumarins, flavones, chromones, and xanthones, while not much was known on polyphenolic acetates (PAs). The work carried out in our laboratory for the first time documented the unique biological actions of PAs not exhibited by their parent polyphenols. These investigations implicated the role of the acetyl moieties of PAs in eliciting the biological actions. The only phenolic acetate known to have pharmacological action has been aspirin and related drugs for their ability to acetylate cyclooxygenase nonenzymatically leading to cessation of prostaglandin synthesis [2]. Our further investigations indeed demonstrated the acetylation of glutathione S-transferase (GST-3-3) by 7,8-diacetoxy-4-methylcoumarin (DAMC), a model PA catalyzed by membrane-bound enzyme termed acetoxy drug: protein transacetylase (TAase) leading to irreversible inhibition of GST activity. Several other enzyme proteins such as NADPH cytochrome c reductase, cytochrome P-450–linked mixed function oxidases (MFOs) and nitric oxide synthase (NOS) were shown to be substrates for TAase-catalyzed acetylation by DAMC, culminating in the modification of their catalytic activities [3–8]. Recently, we have purified TAase to homogeneity from rat liver and human placenta [9,10]. The N-terminal amino acid sequence analysis of TAase when aligned with nonredundant Swiss-Prot Database sequence revealed a perfect match with N-terminal sequence of mature CRT, a prominent Ca^{2+} binding protein of endoplasmic reticulum (ER) [11]. Further studies confirmed the identity of TAase with CRT and led to the designation of TAase as the CRTAase describing the transacetylase function of CRT. The specificity of CRTAase to various classes of PAs with special reference to the position of the acetoxy group with respect to the oxygen heteroatom in the coumarin nucleus and the indispensability of C=O group to the TAase activity have been established by us [12,13]. In this paper, we have elaborated for the first time the participation of acetyl group of 7-acetamido-4-methylcoumarin (7-N-AMC) in CRTAase-catalyzed transfer of acetyl group to proteins and also the potentiation of TAase activity when 7-acetoxy-4-methylthiocoumarin (7-AMTC) was used as the substrate for CRTAase. Further, the CRTAase-catalyzed irreversible inhibition of PKC by PAs in the lymphocytes of asthmatic patients has been correlated with the severity of the disease.

RESULTS AND DISCUSSION

The in-depth studies carried out in our laboratory characterized the CRTAase-catalyzed transfer of acetyl group from the acetoxy derivatives of coumarins, chromones, flavones, isoflavones, and xanthones to functional proteins [12,13]. Efforts were made to examine whether CRTAase could catalyze the transfer of acetyl group from acetamidocoumarin (Fig. 1).

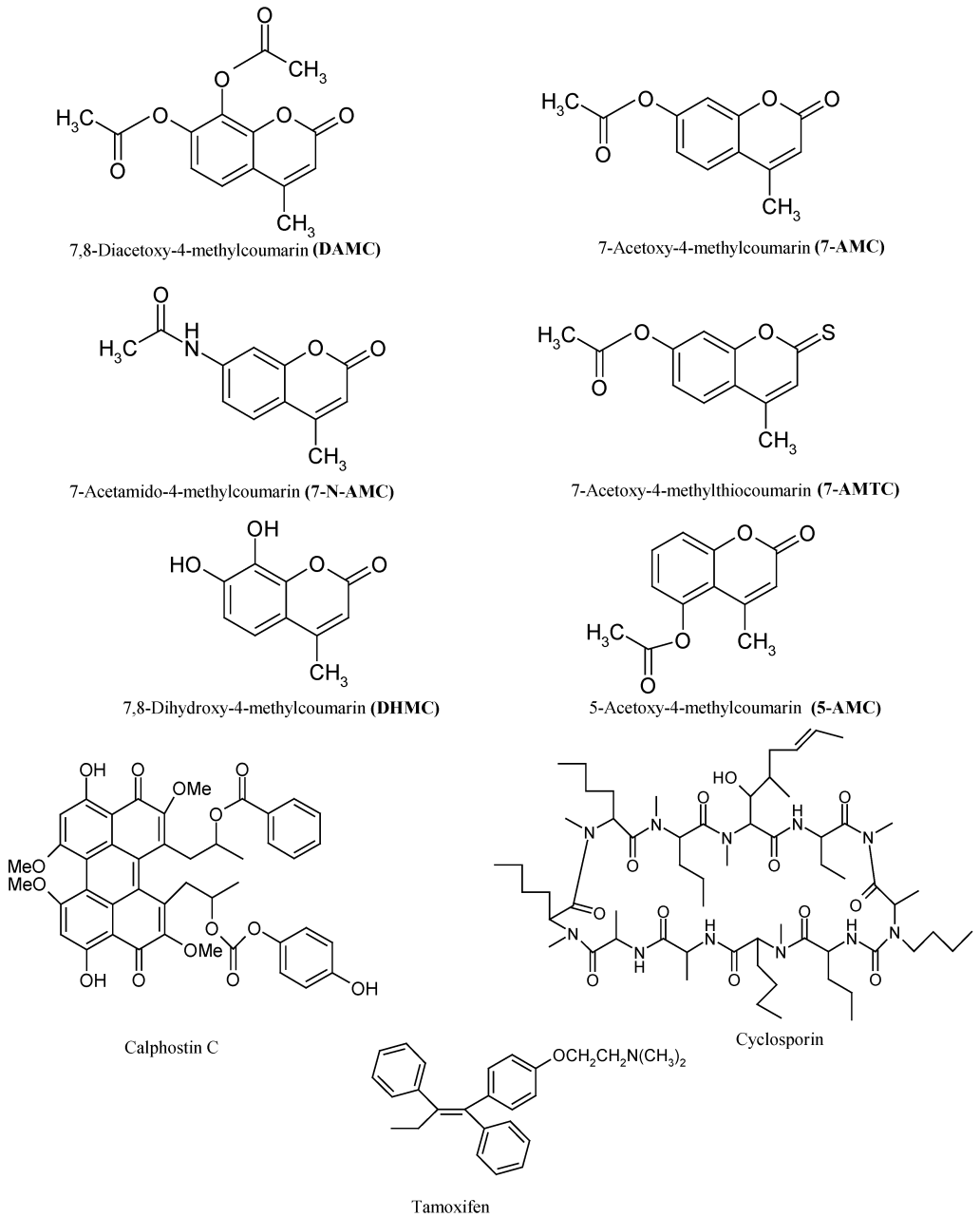


Fig. 1 Structures of polyphenol, polyphenolic acetates, and known inhibitors of PKC.

The results shown in Fig. 2 reveal the specificity of human platelet TAase. It is clear that 7-N-AMC is as effective as monoacetoxycoumarin (7-AMC) when used as substrate for platelet CRTAase. We have also investigated the effect of replacing the C-2 carbonyl group on pyran ring with the >C=S of acetoxycoumarin (Fig. 1) on CRTAase activity.

The results shown in Fig. 2 reveal that C=S group greatly potentiates the CRTAase activity. The 7-AMC exhibits 50 % of CRTAase activity as compared to the activity of DAMC. But the 7-AMTC is almost as effective as DAMC.

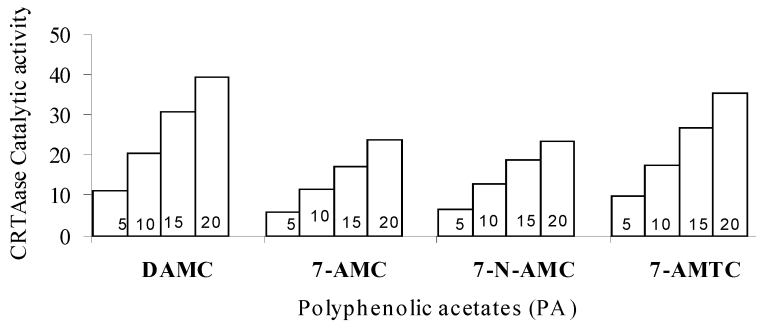


Fig. 2 Comparison of relative specificities of various PAs to platelet CRTAase. CRTAase activity obtained after preincubation with PA at 5, 10, 15, and 20 min (indicated inside the bar) is expressed in terms of % inhibition of GST under conditions of the assay [3]. Concentration of test compounds and platelet lysate proteins was 50 μM and 50 μg, respectively. Values are mean of four observations with variations <5 %.

The relative specificities of platelet CRTAase to activate NADPH cytochrome c reductase by 7-N-AMC and 7-AMC were also compared (Fig. 3). Both these compounds were found to be effective in causing the activation of the reductase to the same extent. Our earlier studies characterized TAase-catalyzed activation of liver microsomal NADPH cytochrome c reductase [4] by DAMC as hyperbolic activation. NOS is a multicomponent enzyme which bears a reductase domain [14]. PAs were found to be effective in causing the activation of NOS catalyzed by CRTAase [8]. The results presented in Figs. 4 and 5 demonstrated that the abilities of 7-N-AMC and 7-AMTC to activate NOS are in tune with their effectiveness to activate the reductase (Fig. 3). The influence of 7-AMTC on NO-related physiological effects, such as platelet aggregation, was examined. It is evident from Fig. 6 that 7-AMTC is as effective as DAMC in inhibiting ADP-induced platelet aggregation.

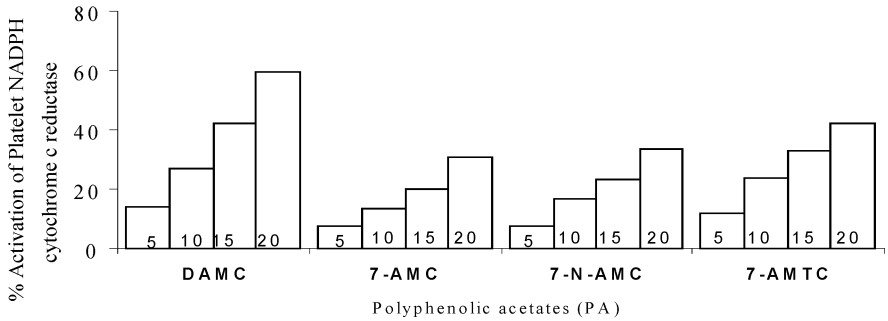


Fig. 3 Activation of platelet NADPH cytochrome c reductase by various PAs catalyzed by CRTAase. The CRTAase-catalyzed activation of NADPH cytochrome c reductase was assessed by preincubation of platelet lysate at 5, 10, 15, and 20 min (indicated inside the bar) with various PAs, followed by the addition of NADPH and cytochrome c in order to assay the reductase activity [4]. The increment in reductase activity over the control (without PA) was considered as the measure of activation. Concentration of test compounds was 25 μM. Values are mean of four observations with variations <2 %.

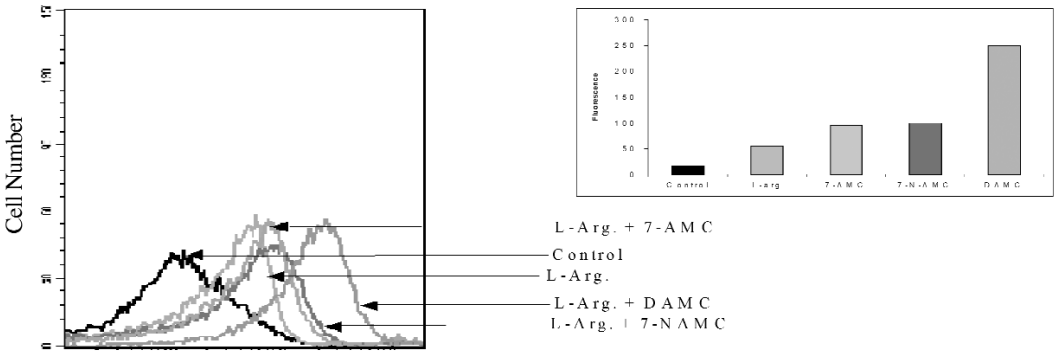


Fig. 4 Comparison of the activation of platelet NOS by DAMC, 7-AMC and 7-N-AMC. Platelets were preincubated along with the test compounds and L-arg. followed by flow cytometric measurement of fluorescence due to DCFH-DA [8]. Concentrations of DAMC, 7-AMC, and 7-NAMC were 100 μM. Sample containing the vehicle (dimethyl sulfoxide, DMSO) alone in place of the test compound served as a control. Values are mean of four experiments with variation less than 4 %.

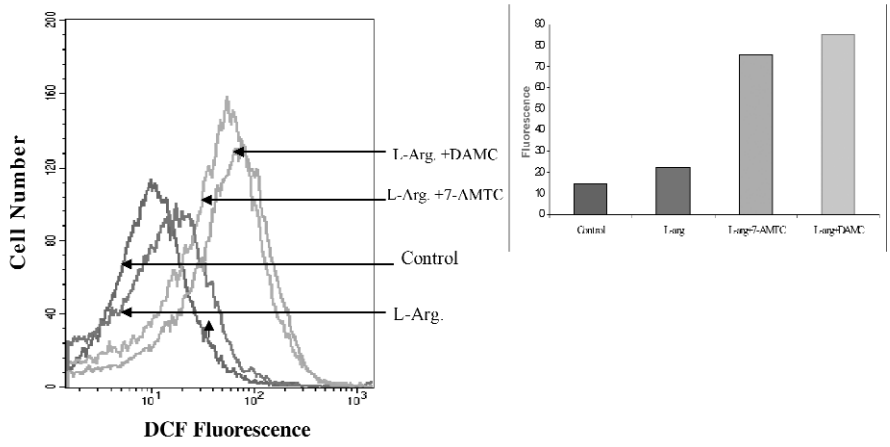


Fig. 5 Comparison of the activation of platelet NOS by DAMC and 7-AMTC. Platelets were separately incubated with test compounds and other components of the assay mixture as described earlier [8]. Concentration of DAMC and 7-AMTC was 100 μM. Sample containing the vehicle (DMSO) served as control. Values are mean of four experiments with variation less than 4 %.

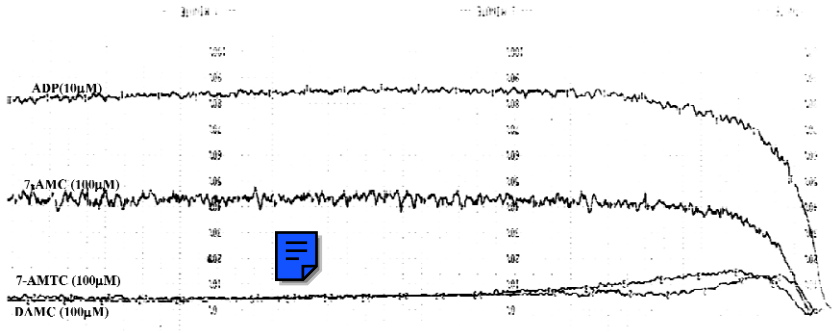


Fig. 6 Comparison of inhibition of ADP-induced platelets aggregation [8] by 7-AMTC, 7-AMC, and DAMC.

The intense fluorescence produced by 7-AMTC in platelets compared to control confirms the remarkable activation of NOS as shown by confocal microscopy (Fig. 7).

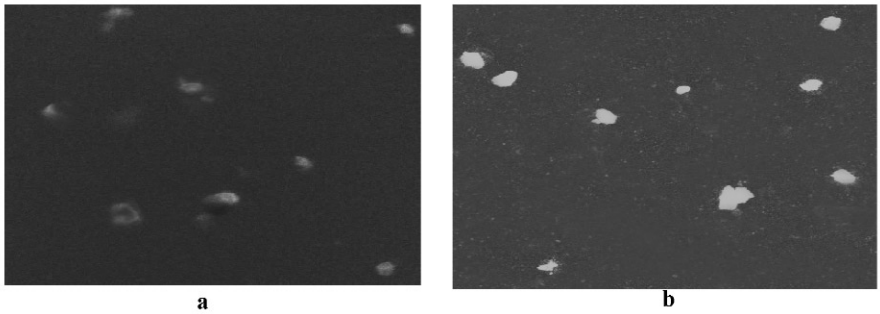
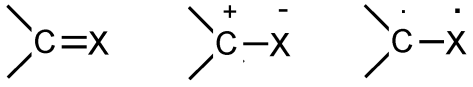


Fig. 7 Demonstration by confocal microscopy [8]: Enhanced production of NO in platelets by 7-AMTC. (a) Platelet was incubated with DMSO. (b) Platelet was incubated with 7-AMTC.

The potentiation of CRTAase activity due to the >C=S moiety on the benzopyran ring of 7-AMTC as compared to 7-AMC is explained by PM3-optimized geometries (Fig. 8), the PM3-optimized geometries revealed that greater TAase activity of 7-AMTC as compared to that of 7-AMC (its oxygen analog) is mainly due to the diffused and polarizable nature of the lone pair of electrons in sulfur and longer >C=S bond lengths [15,16]. The C=S bond length measured (1.61386 Å) for the PM3-optimized geometry of 7-AMTC was found to be larger than that of the C=O bond of 7-AMC (1.22918 Å). This can be explained in terms of the following structural forms:



The structures **b** and **c** contribute more to the thiocarbonyl (X=S) group than to the carbonyl group (X=O), whereas **a** contributes more to the carbonyl group which may be ascribed to the greater π-bond energy for the carbonyl group than that for the thiocarbonyl group. Thus, the availability of the electron pair on the sulfur atom of the thiocarbonyl group of the thiocoumarin may possibly make binding more favorable to the active site of the enzyme as compared to its oxygen analog.

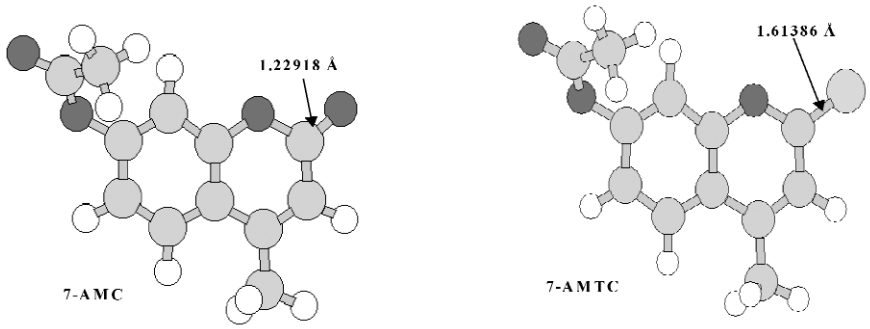


Fig. 8 Optimized molecular structures of 7-AMC and 7-AMTC as obtained by PM3 calculations.

Our work has highlighted the importance of acetyl transferase action of CRT in the modification of functional proteins [9,10] utilizing acetyl group donors such as PA. Asthma is characterized by airway inflammation and bronchial hyper-responsiveness [17]. Several cell types such as lymphocytes, neutrophils, etc. produce inflammatory response by release of several mediators. Also, an enhanced level of PKC is observed in the lymphocytes of asthmatic patients [18]. An effort was made to examine whether lymphocyte PKC could be a target for CRTAase-catalyzed acetylation by PA with a view that alteration of PKC in asthmatic patients could be of therapeutic importance. CRTAase was identified in human peripheral blood lymphocytes. The study included 40 patients and 10 normal subjects. Out of these patients, 10 were taken from each group of asthma, viz. mild intermittent, mild persistent, moderate persistent, and severe persistent. After clinical examination and performance of pulmonary function tests (PFTs), blood samples were withdrawn and lymphocytes were isolated to assay the activity of PKC and CRTAase. The kinetics of CRTAase was carried out using human peripheral blood lymphocytes of normal and asthmatic patients. The lymphocytes of normal controls exhibited K_m and V_{max} values 1818 μM and 175 units, respectively, while similar values in lymphocytes of asthmatic patients were 1666 μM and 200 units, respectively. The differences in kinetic parameters of normal and asthmatic patients were not found significant. We then set out to examine whether lymphocyte PKC is modified by CRTAase-mediated action of DAMC (Fig. 9).

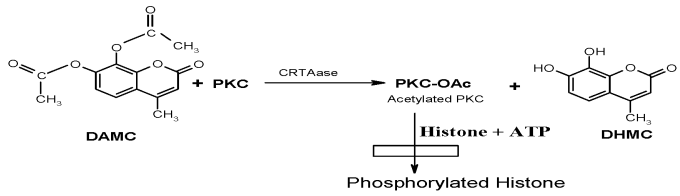


Fig. 9 CRTAase-catalyzed inhibition of PKC by DAMC. PKC was found irreversibly inhibited by DAMC catalyzed by lymphocyte CRTAase. The inhibition of PKC is possibly due to acetylation. Since CRTAase-catalyzed reaction shown above is bimolecular in nature, the enhanced levels of PKC in lymphocytes of asthmatic patients leads to greater inhibition of PKC by DAMC (Fig. 10). □: Denotes inhibition of PKC catalytic activity.

For this purpose, the lymphocyte lysates were preincubated with DAMC (100 μM), followed by the assay of PKC by histone phosphorylation [19]. DAMC was found to cause time-dependent inhibition of PKC (Fig. 10).

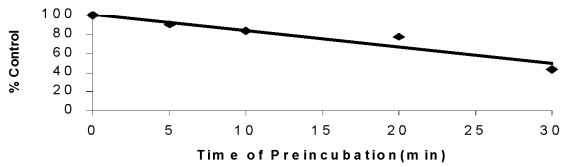


Fig. 10 Lymphocyte CRTAase-mediated time-dependent inhibition of PKC by DAMC. Lymphocyte lysates of normal subjects were used for the assay of PKC by histone phosphorylation method [19]. The concentration of DAMC was 100 μM in the preincubation reaction mixture.

The action of several PAs on the lymphocyte PKC activities of normal and asthmatic patients was investigated. The activity of PKC increased at every stage of asthma. The results presented in Fig. 11 point out that PA caused inhibition of PKC of lymphocytes of normal patients marginally. On the other hand, PA caused inhibition of lymphocyte PKC of asthmatic patients drastically (Fig. 11). PA-produced inhibition of lymphocyte PKC was in tune with their specificity to CRTAase, viz. DAMC > 7-AMC >

5-AMC > DHMC. It is noteworthy that CRTAase-catalyzed inhibition of PKC is seen as significant only in cases of moderate persistent and severe persistent asthma (Fig. 11).

Monoacetoxycoumarin, such as 5-AMC which is a poor substrate for CRTAase, was the least effective in causing the inhibition of PKC (Fig. 11). DHMC, the deacetylated product of DAMC, was totally ineffective in causing the inhibition of PKC (Fig. 11). These observations amply purport the role of CRTAase mediation in the inhibition of PKC by PA. Several inhibitors of PKC, such as calphostin C, tamoxifen, cyclosporine, etc. (Fig. 1), are known [20,21]. Most of these are competitive inhibitors of PKC that cause inhibition by binding to the regulatory or the catalytic domain of PKC [21]. The results presented here highlighted for the first time that DAMC is an irreversible inhibitor of PKC by possible acetylation of this protein. There have been no reports on the modification of PKC by way of acetylation. The inhibition of PKC is known to result in diminished release of mediators of inflammation, such as IL-2 [22]. Several PAs through their action as discussed above can be expected to play an important role in controlling the inflammation in asthmatic conditions and may eventually serve as potential drug candidates.

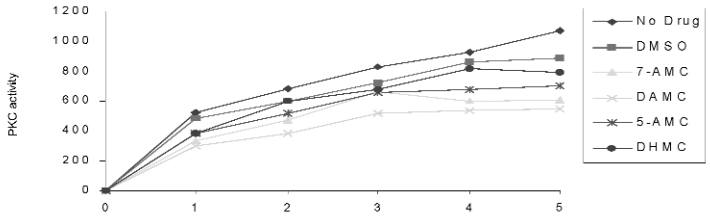


Fig. 11 Effects of several PAs on lymphocyte PKC activity of normal subjects and patients at various stages of asthma. Lymphocyte PKC was assayed by histone phoshorylation. The activity of PKC was expressed as f moles of ^{32}P-transformed to histone per mg protein under experimental conditions. Concentration of the test compounds was 100 μM in the preincubation reaction mixture.

	MI	MP	Mo.P	SP
DAMC	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$
7-AMC	NS	NS	$P < 0.001$	$P < 0.001$
5-AMC	NS	NS	$P < 0.05$	$P < 0.001$
DHMC	NS	NS	NS	NS

1: normal; 2: mild intermitent (MI); 3: mild persistent (MP); 4: moderate persistent (Mo. P); 5: severe persistent (SP).

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