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APOPROTEIN A-1 IS A COFACTOR INDEPENDENT SUBSTRATE OF PROTEIN KINASE C

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Apoprotein A-1 (apo A-1), the predominant protein constituent of high density lipoproteins (HDL), was phosphorylated by protein kinase C (PKC). Optimal phosphorylation of lipid-free apo A-1 occurs in the absence of calcium, phosphatidyl serine (PS), and diolein (DO). However, HDL-bound apo A-1 was not phosphorylated by PKC. Furthermore, addition of either native or reconstituted HDL particles to lipid-free apo A-1 resulted in a concentration-dependent inhibition of phosphorylation. It appears that the phosphorylatable sites on apo A-1 are involved in hydrophobic interaction with the lipids of HDL. Apo A-1 is a novel substrate of PKC because it does not require calcium and lipid cofactors for optimal phosphorylation.

High density lipoproteins (HDL) promote the efflux of cholesterol from peripheral tissues and its delivery to liver and steroidogenic tissues (1). Epidemiological data indicate that plasma levels of HDL cholesterol levels are inversely proportional to the incidence of coronary heart disease. (2,3) Therefore, it has been postulated that HDL exert a protective effect on coronary heart disease. Apoprotein A-1 (apo A-1) is the predominant protein constituent of high density lipoproteins. It functions as a cofactor for lecithin-cholesterol acyltransferase (LCAT) catalyzed esterification of cholesterol (4) and has also been implicated as the ligand for HDL - receptor recognition. (5,6) The existence of specific HDL receptors has been theorized because of numerous experimental demonstrations of the binding of HDL to cells (7-10). Although the binding of apo A-1 to cytosolic and plasma membrane proteins has been demonstrated in several cell types (6,11), there have been no data on the interaction of apo A-1 with a specific cellular trans-membrane signalling mechanism.

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In the present communication we describe the phosphorylation of apo A-1 by protein kinase C (PKC). This enzyme is a key member of cellular signal transduction system (12). When histones are used as PKC substrates, total expression of activity is achieved only in the presence of calcium, phospholipids and diacylglycerol. Phosphorylation of apo A-1 by PKC however, is accomplished in the absence of calcium and lipid cofactors. This cofactor-independent phosphorylation by PKC of a physiologically relevant substrate is a novel finding regarding the action of this enzyme, and may have significant bearing on the interaction of apo A-1 with cellular components and on its function.

MATERIALS AND METHODS

Apo A-1, HDL, phosphatidyl serine (PS), diolein (DO), histones (type III-S), and H-7 were purchased from Sigma Chem. Co.; γ-^{32}P-ATP was from DuPont - New England Nuclear. DEAE-cellulose (DE-52) was obtained from Whatman, while Sephadex G-200 and phenyl Sepharose were from Pharmacia; low MW markers were purchased from Bio Rad. PKC and cAMP-dependent protein kinase (cAMPdPK) were isolated from rat brain as described below.

Isolation of cAMPdPK

Fresh rat brain was homogenized in 7 volumes of 20 mM Tris buffer pH 7.5, and the homogenate centrifuged at 20,000g for 20 min. The supernatant was applied to a DE-52 column (1.5 cm x 10 cm), the column was washed with 10 volumes of the above Tris buffer and eluted with an 80 ml gradient of 0 to 0.4 M KCl in Tris buffer. The second peak of cAMP-dependent activity was pooled, concentrated by ultrafiltration and used as the cAMPdPK preparation. cAMPdPK activity was monitored by measuring the transfer of ^{32}P from γ^{32}P-ATP into histones in the absence and presence of 5 μM cAMP as previously described (13).

Isolation of PKC

During PKC preparation all operations were carried out at 4°C. Highly purified PKC was prepared by sequential ion exchange, molecular sieve and affinity chromatography. Fresh or freshly frozen rat brain, weighing approximately 2.0 g, was homogenized in 7 volumes of 20 mM Tris buffer pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 10 mM mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 20 min at 20,000 g and the supernatant applied to a DE-52 column (1 cm x 6.5 cm). The column was washed with 20 volumes of the above Tris buffer and was eluted with a 20 ml gradient of 0 to 0.3 M KCl in the buffer. The PKC containing fractions were pooled, concentrated by ultrafiltration and applied to a Sephadex G-200 column (1.5 cm x 19 cm). The column was eluted with the Tris buffer and PKC fractions were pooled again, concentrated, and adjusted to 1.5 M NaCl. This sample was applied to a Phenyl-Sepharose column (0.7 cm x 1 cm) and the column washed stepwise with a) 5 ml of 1.5 M NaCl in the Tris buffer, b) 2 ml of 1.0 M NaCl in buffer, c) 2 ml of 0.5 M NaCl in buffer, and PKC was eluted with 2 ml of salt-free Tris buffer. The PKC thus isolated was nearly homogeneous based on Coomassie blue staining following SDS-PAGE. Histone phosphorylation by this PKC preparation was dependent on calcium, PS and DO.

Throughout the purification procedure PKC activity was monitored by the transfer of ^{32}P from γ^{-32}P-ATP into lysine-rich histones as previously described (14).

Several PKC preparations were used during this investigation, with specific activities ranging between 10,000 and 20,000 pmoles/mg prot/min.

Substrate Phosphorylation

PKC catalyzed phosphorylation of substrates was carried out at 30°C for specified time in 75 μl of a reaction mixture containing: 20 mM Tris buffer pH 7.5, 6 mM $MgCl_2$, 33 μM γ-^{32}P-ATP (sp. act. 1500 to 2500 cpm/pmole), apo A-1 (0.62 to 10 μg) or HDL (2 to 20 μg), PKC (2 to 10 μg) and, where indicated, lipid cofactors. When cAMPdPK was used as the phosphorylating enzyme in place of PKC, 16 μg of the cAMPdPK preparation were added. The reaction was terminated by the addition of 25 μl of a stopping solution (15% sucrose, 30 mM Tris pH 8.0, 3 mM EDTA, 6% SDS, 0.25% Bromphenol blue, and 75 mg/ml DTT added fresh) and heating at 100°C for 5 min.

Protein components were separated by SDS-PAGE using 5% stacking gel and 10% separating gel according to the procedure of Laemmli (15). ^{32}P labelling was identified by autoradiography on Kodak X-O mat AR film and was quantitated either by densitometry or by excising the specified protein band from a dried gel and determining the incorporated radioactivity by liquid scintillation counting.

Other Procedures

Protein concentration was determined by the method of Lowry et al (16). Reconstituted HDL were prepared according to the method of Jonas et al (17).

RESULTS AND DISCUSSION

PKC catalyzed phosphorylation of histones and several other substrates requires calcium and phospholipid vesicles for full expression of enzyme activity (18,19). It was, therefore, of particular interest to investigate whether a protein that is integrally associated with phospholipid containing particles behaves as a substrate for PKC, and whether the associated phospholipids exert a regulatory function on its phosphorylation. These initial experiments were carried out to determine whether apo A-1, the primary protein constituent of HDL particles, is phosphorylated by purified rat brain PKC.

Lipid free apo A-1 migrates on SDS-PAGE as a single protein band of approximately 27 kDa. Results presented in Fig. 1 show that the 27 kDa apo A-1 protein is phosphorylated by PKC in a concentration-dependent fashion. This phosphorylation, however, was not dependent on the presence of calcium and the lipid cofactors, PS and DO. In fact, in their presence, there was a partial inhibition of phosphorylation which seemed to be more pronounced at lower concentrations of apo A-1 (Fig. 1). The time course of apo A-1 phosphorylation by PKC in the absence of calcium, PS and DO is depicted in Fig. 2. Results show a very rapid initial rate of phosphorylation that moderates after the first minute of incubation but does not reach a plateau even after 20 minutes of incubation at 30°C.

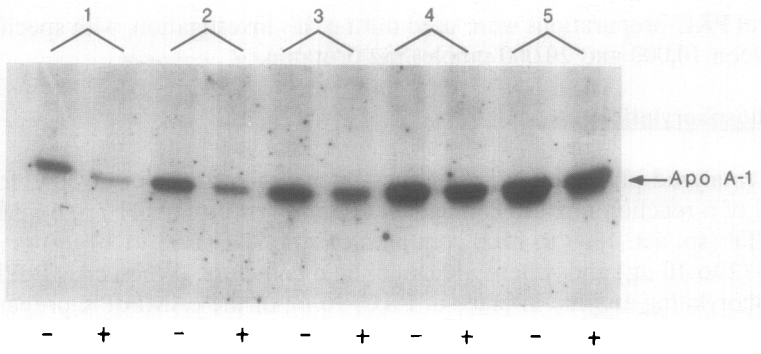


Fig. 1. Autoradiogram depicting the phosphorylation of apo A-1 by PKC. Increasing amounts of apo A-1 (1: 0.625 μg, 2: 1.25 μg, 3: 2.5 μg, 4: 5.0 μg, and 5: 10.0 μg) were incubated with PKC for 5 min at 30°C in the absence (-) or presence (+) of 0.5 mM calcium, 50 μg/ml PS and 1 μg/ml DO as described in detail under Materials and Methods.

Having observed that lipid free apo A-1 is phosphorylated by PKC, we subsequently investigated the phosphorylation of HDL-bound apo A-1. Results (Fig. 3, lane 1) indicate that when HDL were used as substrate the apo A-1 constituent in HDL particles was not phosphorylated by PKC, even though the amount of apo A-1 within HDL was greater than the amount of lipid-free apo A-1 that was phosphorylated by PKC (Fig. 3, lane 3). To establish whether the inability of PKC to phosphorylate HDL-associated apo A-1 was due

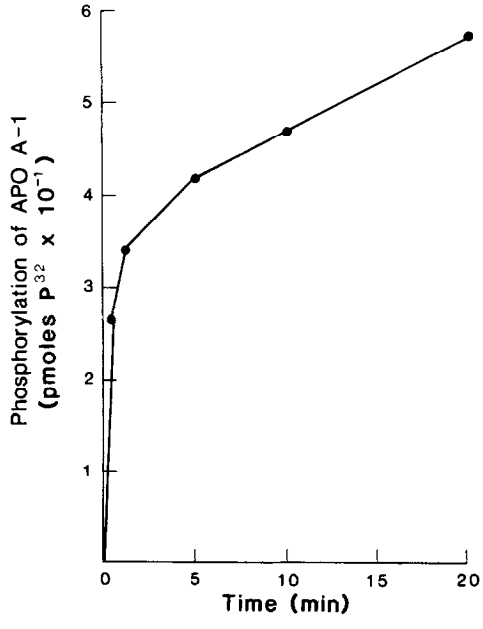
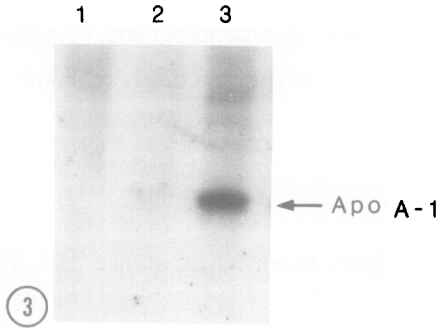


Fig. 2. Time course of phosphorylation of apo A-1 by PKC in the absence of calcium, PS and DO. The rate of phosphorylation was determined by counting the radioactivity in excised gel pieces corresponding to the (27 kDa) apo A-1 protein band.



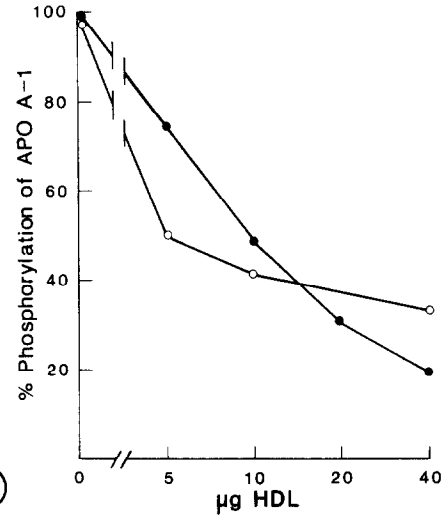


Fig. 3. Autoradiogram of the SDS-PAGE of a reaction in which phosphorylation was performed using the following substrates: (1) 10 µg of native HDL, (2) 10 µg of rHDL, and (3) 1.25 µg of lipid-free apo A-1.

Fig. 4. Effect of increasing concentrations of native (●) or rHDL (○) on PKC catalyzed phosyphorylation of lipid-free apo A-1; 1.25 µg of apo A-1 were incubated for 5 min at 30°C with increasing concentrations of the respective HDL preparations. Phosphorylation of the apo A-1 band from SDS-PAGE was quantitated by densitometric measurement of the autoradiogram.

to the natural components of HDL or was due to an impurity which copurified with the HDL particles, we prepared pure reconstituted HDL (rHDL) and examined their ability to serve as PKC substrates. As seen in Fig. 3, lane 2, the apo A-1 component of rHDL was also not phosphorylated by PKC. It is, therefore, reasonable to conclude that the lipid/phospholipid components of HDL exert an inhibitory effect on apo A-1 phosphorylation by PKC.

The HDL preparation used in the current investigation was a mixture of several HDL subfractions and was not saturated with apo A-1. Such preparations have the ability to bind additional molecules of apo A-1. We, therefore, studied the effect of native HDL preparations and also that of rHDL on phosphorylation of lipid-free apo A-1. Results presented in Fig. 4 show that addition of either of the HDL preparations suppresses PKC mediated phosphorylation of lipid-free apo A-1 in a concentration-dependent manner. It is speculated that the inhibition observed probably results from a masking of serine and threonine residues (available for phosphorylation) due to hydrophobic interactions occurring between apo A-1 and the phospholipids of HDL.

The identification of apo A-1 as a PKC substrate is significant in that it suggests a role for PKC mediated signal transduction in apo A-1 function and thus in cholesterol transport and/or its metabolism. The finding that PKC mediated phosphorylation of apo A-1

is independent of calcium and lipid cofactors identifies it as a new type of substrate for PKC. The inhibition exerted by HDL suggests a novel regulatory function which appears to be self-contained within the HDL particle. This observation also implies that under physiological conditions, apo A-1 would be phosphorylated only after it has dissociated from the HDL particle. Such dissociation might take place subsequent to the interaction of HDL with their specific receptors and, therefore, phosphorylation of apo A-1 might be a direct outcome of HDL receptor occupancy.

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