cAMP MEDIATED DECREASE IN MEMBRANE PHOSPHORYLATION

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SUMMARY

An endogenous cAMP independent protein kinase incorporated label from (γ-^{32}P)-ATP into a 58,000 and a 55,000 dalton component in the microsomal membrane fraction from rat adipocytes. An endogenous cAMP dependent protein kinase incorporated label into a 19,000 and a 15,000 dalton component only in the presence of cAMP. An unexpected result of the presence of cAMP was a marked decrease in the cAMP independent protein kinase labeling of the higher molecular weight components. This decrease was eliminated by using higher levels of ATP which implies that the two kinds of kinases compete for ATP in the presence of cAMP. The possible physiological importance of this competition which results in decreased phosphorylation by cAMP independent protein kinases in the presence of cAMP is considered.

INTRODUCTION

Using high energy phosphate compounds as donors, kinases phosphorylate proteins and thereby often modify the activity of enzymes. Finding that cAMP dependent protein kinases were widely distributed in nature, Kuo and Greengard (1, 2) hypothesized that they might mediate all of the physiological effects of cAMP. The recent report (3) of a cAMP dependent protein phosphatase which removes phosphate from phosphorylated proteins suggested another way cAMP may mediate its physiological effects. This paper reports that cAMP decreases the cAMP independent phosphorylation of proteins by activating cAMP dependent kinases which compete for available ATP. This decreased phosphorylation may be another way some of the physiological effects of cAMP are mediated.

Abbreviations used: cAMP, adenosine 3',5'cyclic monophosphoric acid; ATP, adenosine 5'-triphosphoric acid; SDS, sodium dodecyl sulphate; P_i, inorganic phosphate.

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MATERIALS AND METHODS

Sodium dodecyl sulfate (SDS) was recrystallized from ethanol while acrylamide was recrystallized from chloroform. The bovine serum albumin (Sigma Chemical Co.) was essentially fatty acid-free and prepared from fraction V albumin. The albumin was dialyzed exhaustively against Krebs-Ringer solution (4) with 1/2 the recommended Ca^{++} before use. Crude collagenase, Type I from *clostridia histolyticum*, was obtained from Worthington Biochemical Corp. New England Nuclear supplied (γ-^{32}P)-ATP at 10-50 Ci/mmol with greater than 97% purity. All other chemicals used were reagent grade and were obtained from common suppliers.

Fat cells were prepared from the epididymal fat pads of male Sprague-Dawley rats as described by Rodbell (5) except that only 5 mgm of collagenase were used per gram of fat and glucose was omitted. Following the digestion, individual cells were passed through a nylon cloth with 200 µm openings. The cells were washed at 37°C 5 times by centrifuging and discarding the infranatant and pellet. The first wash was with Krebs-Ringer bicarbonate buffer containing 4% albumin. The next three washes were with Krebs-Ringer phosphate buffer (92 ml of Krebs-Ringer solution (4) [using 1/3 the Ca^{++}] plus 8 ml of 0.1 M Na_2HPO_4) at pH = 7.4. The last wash was with 0.25 M sucrose and 10 mM Tris (hydroxymethyl) aminomethane adjusted to pH = 7.4 at 25°C with HCl. The washed cells were then suspended in the latter buffer and disrupted according to the procedure of Avruch and Wallach (6). The cell suspension was cooled to zero degrees and rapidly passed through a 48 µm mesh nylon cloth between two stainless steel support screens with 200 µm photo-etched holes in a Swinney filter holder. The homogenate was warmed to 37°C and centrifuged at 30,000 g for 10 min. The infranatant and pellet were collected and processed to isolate the microsomal fraction according to Avruch and Wallach (6). A 35% (wt./wt.) sucrose solution was substituted for the continuous sucrose gradient (7, 8) and the microsomes were washed at pH = 7.4 instead of 8.6.

Protein was determined according to Lowry *et al.* (9) using bovine serum albumin as a standard while 5'-nucleotidase activity was measured according to Avruch and Wallach (6). The microsomal membrane fraction was phosphorylated at pH = 7.0 at 25°C in a solution which was 0.01 M in Na_2HPO_4, 1 mM in $MgCl_2$, 0.14 M in NaCl and 0.3 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetracetic acid. The membranes were incubated at 25°C for 15 min. prior to starting phosphorylation by adding labeled ATP. When cAMP was present, it was added simultaneously with theophylline to yield a final concentration of 2 µM cAMP and 2 mM theophylline. Following incubation at 25°C for the required time, the reaction was stopped by adding an equal volume of an SDS sample buffer which is twice as concentrated as the "final sample buffer" described by Laemmli (10). SDS gel electrophoresis was performed as described by Laemmli (10) in slab gels of 1.5 mm thickness. The running gel was 10 cm long and consisted of a gradient of acrylamide from 7.5% at the top to 17.5% at the bottom. Proteins were visualized in gels by staining with Coomassie Brilliant Blue. Molecular weights were determined by comparison with the position of proteins of known molecular weights which were electrophoresed on the same gel. Radioactive components were detected by autoradiography of vacuum dried gels. Kodak X-Omat R Film (XR-5) was used. Autoradiographs were scanned on an Ortec 4310 densitometer.

RESULTS AND DISCUSSION

The analysis of our microsomal membrane fraction compares favorably with other reports (6, 7, 11). The specific activity of 5'-nucleotidase averaged over four of our membrane preparations is 4.1 µmoles/hour/mgm protein. This

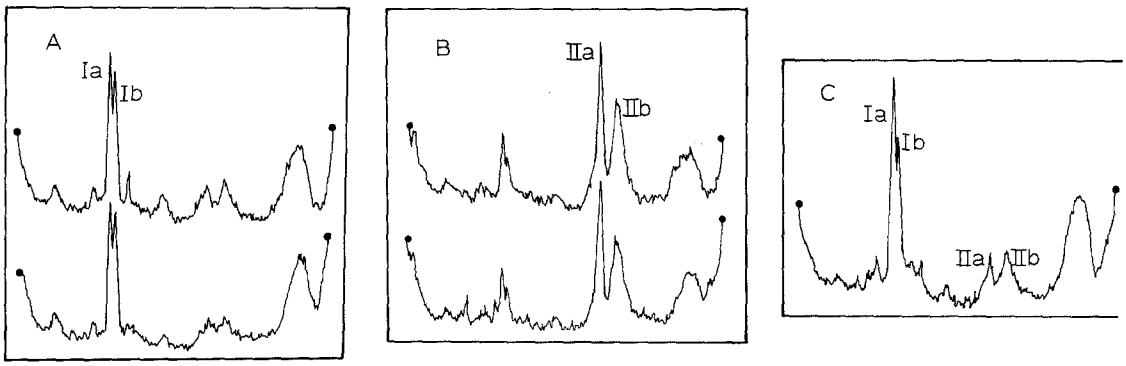

Figure 1 - Densitometer tracings of autoradiographs of dried SDS slab gels on which were electrophoresed microsomal membranes exposed to 10 μM (γ-^{32}P)-ATP for 15 sec. (lower tracing) or 30 sec. (upper tracing). The lowest point in each tracing is coincident with the base line. A, absence of cAMP; B, presence of cAMP; C, 15 sec. in the absence of cAMP followed by 15 sec. in the presence of cAMP.

represents a 5.4-fold increase in specific activity when compared to the whole cell homogenates.

Figure 1 shows how the ^{32}P was distributed on the SDS gels following electrophoresis of membranes phosphorylated by endogenous kinases using added (γ-^{32}P)-ATP. The distributions were not altered by chasing the label with a large excess of cold ATP prior to solubilizing the membranes for electrophoresis. Hence, the label was incorporated into long-lived species. Heating the gel to 90°C in 10% trichloroacetic acid for 35 min. also did not affect how the label was distributed. Since this treatment has been reported (11) to solubilize nucleic acids from the gel, the label is probably in phosphopeptides and phospholipids. The protein distribution following electrophoresis on SDS-gels was not affected by the time of incubation or by the presence or absence of cAMP or ATP. Figure 1A shows how the label was distributed when the membranes were phosphorylated in the absence of cAMP. The peaks marked Ia and Ib have molecular weights of 58,000 and 55,000 daltons, respectively. In the presence of cAMP, peaks marked IIa and IIb with molecular weights of 19,000 and 15,000 daltons are greatly increased as seen in Figure 1B. Other investigators (12, 13) reported

an increase in nearly identical components and attributed the increase to the action of cAMP dependent protein kinase.

A most striking effect of cAMP on the membrane phosphorylation was the decreased labeling in peak Ia and Ib as seen when Figure 1B is compared with Figure 1A. Since a cAMP dependent phosphatase has been reported (3), we added cAMP to membranes which were initially phosphorylated in the absence of cAMP. The results are shown in Figure 1C. Similar results were obtained when the time of incubation with cAMP was extended from 15 sec. to 2 min. Clearly cAMP does not promote the removal of label from component Ia and Ib once they are labeled. Hence, cAMP must decrease the labeling of components Ia and Ib. This may be due to inhibition of the kinase by cAMP or cAMP could enhance alternative uses of ATP thereby reducing the labeling of components Ia and Ib because ATP is the limiting factor. These possibilities were tested by increasing the initial amount of ATP available for the phosphorylation reaction. With more ATP available, competition for ATP would be reduced whereas inhibition by cAMP would not be changed.

Figure 2A shows the rate of labeling of peaks Ia,b (lower graph) and of IIa,b (upper graph) in the absence and presence of cAMP when the initial ATP concentration is 100 μM. When compared to the rates of labeling in Figure 2B where the initial concentration of ATP is 10 μM, it is clear that the cAMP stimulated increase in labeling of peak IIa,b is nearly identical at the two different ATP concentrations. However, the labeling of peak Ia,b is not decreased as much by cAMP at the higher ATP concentration. Hence, the cAMP independent protein kinase which catalyzes the labeling of peak Ia,b is probably not inhibited by cAMP. Since cAMP neither inhibits the enzyme involved in labeling peak Ia,b nor stimulates the removal of label from the peak, cAMP probably enhances a reaction which competes for the labeled substrate, (γ-^{32}P)-ATP. One likely candidate for this competing reaction is the cAMP dependent protein kinase which labels peak IIa,b. However, another possibility is a cAMP stimulated ATPase. While ATPases are present in most membranes, only an ATPase whose activity is enhanced by cAMP would increase the competition for ATP in the presence of cAMP.

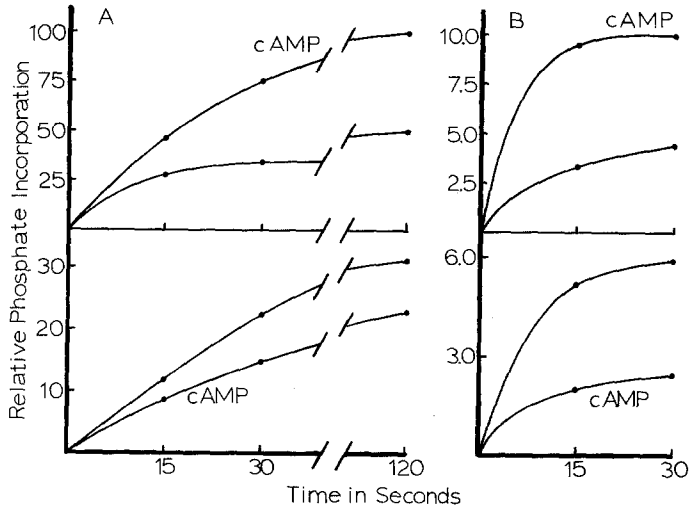

Figure 2 - Relative incorporation of phosphate into peak Ia,b (lower graphs) or into peak IIa,b (upper graphs) determined by weighing the peaks cut out from the densitometer tracings described in the legend to Figure 1 and then taking into account the specific activity and the time in autoradiography. Incorporation into peak IIa,b in the presence of cAMP with 100 μM ATP for 2 min. was taken as 100. A, 100 μM (γ-^{32}P)-ATP; B, 10 μM (γ-^{32}P)-ATP.

Such an ATPase has not been reported in the fat cell. If it were present, the decreased labeling by cAMP independent protein kinases in the presence of cAMP would still be the result of competition for ATP.

Decreased phosphorylation by cAMP independent kinases in the presence of cAMP because of competition for ATP by cAMP dependent kinases may be a physiologically important response to increased intracellular levels of cAMP. Increased levels of cAMP could lead to increased phosphorylation of some proteins and decreased phosphorylation of others. To be physiologically important, the decreased phosphorylation mediated by cAMP should be observed in the intact cell. In the intact fat cell, there is one report (14) of a cAMP mediated decrease in phosphorylation and two reports (15, 16) where a decrease was not found. Similarl there is one report of such a decrease for the intact red blood cell (17). However, such results in intact cells are difficult to interpret because of the action of endogenous phosphatases. The most cogent argument for the physiologica

importance of competition for ATP between cAMP independent and dependent protein kinases comes from the work of Chandramouli *et al.* (18). They found that reducing the ATP levels of intact fat cells to 10-40% of normal abolished insulin stimulated glucose uptake. They suggested that high-energy phosphate bonds are necessary for insulin stimulation of glucose transport, probably via phosphorylation-dephosphorylation of membrane proteins. Hence, an activated kinase which consumes and thereby reduces an intracellular pool of ATP to 10-40% of normal may significantly decrease the phosphorylation by another non-activated kinase.

The greatly increased phosphorylation of the 19,000 and 15,000 dalton components of the isolated membrane in the presence of cAMP is not observed when dibutyrl cAMP (or epinephrine) is added to $^{32}P_i$-labeled intact adipocytes. It has been suggested (13) that this difference represents either an altered accessibility of kinase to potential substrates in the isolated membrane or the presence of a protein kinase on the exterior surface of the membrane which cannot use the ATP inside the cell. Indeed, membranes isolated from intact fat cells exposed to (γ-^{32}P)-ATP are labeled predominantly in the 19,000 and 15,000 dalton components (19). However, we have found (unpublished data) that when intact adipocytes are exposed to (γ-^{32}P)-ATP and directly solubilized in SDS and electrophoresed, a 43,000 dalton component is predominantly labeled independent of cAMP with slight labeling in the 19,000 and 15,000 dalton components which depends on cAMP. The reasons for these discrepancies are being investigated.

The 58,000 and 55,000 dalton components of the isolated membrane phosphorylated in the absence of cAMP may coincide with phosphorylated proteins observed by others. Avruch *et al.* (15) observed a 62,000 dalton component that was phosphorylated in intact cells in the absence and in the presence of cAMP. Benjamin and Singer (14) reported a 50,000 dalton component in the intact fat cell whose phosphorylation was increased by insulin and decreased by epinephrine. Forn and Greengard (16) showed a phosphorylated protein in the intact adipocyte of about 55,000 daltons whose phosphorylation was not affected by insulin or nonepinephrine.

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