Proceedings of the National Academy of Sciences
Vol. 67, No. 1, pp. 290–295, September 1970

ATP-Dependent and Cyclic AMP-Dependent Activation of Rat Adipose Tissue Lipase by Protein Kinase from Rabbit Skeletal Muscle*

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Communicated by Clifford Grobstein, June 24, 1970

Abstract. Brief incubation of partially purified preparations of hormone-sensitive lipase from rat epididymal fat pads with ATP, Mg^{++}, cyclic adenosine 3′:5′-monophosphate and rabbit muscle protein kinase (phosphorylase *b* kinase kinase) resulted in enhancement of lipolytic activity (44–93%). Little or no activation was observed when either the cofactor mixture or the protein kinase was omitted. When the fat pads were incubated with epinephrine prior to homogenization, addition of kinase and cofactors to the soluble supernatant fraction caused no activation whereas good activation was obtained in preparations from paired fat pads not exposed to epinephrine. The results indicate that the cyclic AMP-mediated activation of hormone-sensitive lipase in adipose tissue involves a protein phosphorylation step. Whether the lipase itself is phosphorylated and thus activated or whether the protein kinase is activating a mediating enzyme, in analogy with its action in the glycogen phosphorylase system, remains to be determined.

The rate of release of free fatty acids (FFA) from adipose tissue is controlled by regulation of the activity of hormone-sensitive triglyceride lipase.[1] The several hormones that acutely activate this lipase (including catecholamines, adrenocorticotropic hormone, glucagon, and thyroid-stimulating hormone) stimulate adenyl cyclase activity and increase adipose tissue levels of cyclic adenosine 3′:5′-monophosphate (cAMP),[2–4] which is believed to play a "second messenger" role.[5] Lipase activation dependent on ATP and cAMP in cell-free preparations of adipose tissue has been reported by Rizack[6] and by Tsai and Vaughan.[7] However, the role of cAMP and the nature of the steps between cAMP formation and activation of hormone-sensitive lipase remain undefined. The presence of a lipase-inactivating system in crude adipose tissue homogenates[8] and the lack of progress in purification of the enzyme have made studies of the activation process difficult. We recently reported methods for preparation of a stable, particle-free fraction containing hormone-sensitive lipase[9–11] and have now obtained the enzyme, a large lipoprotein molecule, in pure form.[12]

Walsh, Perkins, and Krebs[13] have purified from rabbit skeletal muscle a cAMP-stimulated protein kinase that converts inactive phosphorylase *b* kinase (by ATP-dependent phosphorylation) to the active form (EC 2.7.1.38) which,

in turn, converts phosphorylase *b* to its active form, phophorylase *a* (EC 2.4.1.1). The same protein kinase has recently been shown to phosphorylate the I form of glycogen synthetase, converting it to the D form.[14] We wish to report that a partially purified preparation of this rabbit muscle protein kinase (phosphorylase *b* kinase kinase) also catalyzes an ATP-dependent and cAMP-dependent enhancement of lipolytic activity in cell-free fractions of rat adipose tissue.

Methods. Epididymal fat pads from Sprague-Dawley rats (200–300 g) were incubated for 3–4 hr in Krebs-Ringer bicarbonate, pH 7.4, containing 4% bovine serum albumin. They were then homogenized in 10 vol ice-cold 0.25 M sucrose containing 10^{-3} M EGTA and the homogenates were centrifuged for 30 min at 40,000 rpm in an SW56 rotor using a Beckman L2-65B centrifuge. The cake of fat at the top of the tubes was removed by slicing and the clear fluid decanted from the sediment was designated the S_{100} fraction. Adjustment of pH to 5.2 yielded a precipitate (5.2P fraction) previously shown to contain 90% of the hormone-sensitive lipase in the soluble supernatant fraction and representing a 3- to 6-fold purification.[10, 11] The 5.2P fraction was taken up in 0.02 M Tris buffer, pH 7.4, containing 10^{-3} M EGTA and density was adjusted to 1.12 by further addition of sucrose. After 48 hr centrifugation at 105,000 $\times$ *g* the top fraction, previously shown to contain more than 50% of the lipase activity present in the 5.2P fraction and representing a 15- to 30-fold purification,[11] was removed and designated the $d < 1.12$ fraction.

Lipase activity was assayed as previously described[11] using as substrate an emulsion of [^{14}C]triolein (8 μmol/ml) stabilized with 5% gum arabic. Free fatty acids released were extracted by the method of Kelley.[15,16] Duplicate lipase assays, done on each sample, differed by less than 10% in most cases and the mean values are presented. The rate of release of labeled free fatty acids remained constant over the assay period both in the absence and in the presence of the lipase-activating system.

Partially purified protein kinase was prepared from rabbit skeletal muscle according to Walsh *et al.*,[13] through the first DEAE-cellulose column step (elution with 0.03 M potassium phosphate buffer, pH 7.0) and stored at −65°C. Preparations were thawed and used either at once or after storage at ice temperatures for no more than 6 hr. Adipose tissue fractions (corresponding to 10–50 mg wet weight of tissue) were incubated with protein kinase (60 μg of protein) for 3–6 min at 30°C in the presence of the following complete system (final concentrations): ATP, 10^{-3} M; Mg acetate, 10^{-2} M; cAMP, 10^{-5} M (unless otherwise indicated); theophylline, 2×10^{-3} M; β-glycerophosphate, 0.1 M, pH 6.2; EGTA, 10^{-3} M. With the large amounts of protein kinase activity added, the time of the preincubation did not significantly influence the degree of activation.

Results. As shown by the representative results in Tables 1–3, prior incubation of either the soluble supernatant fraction (S_{100}) or the pH 5.2 precipitate fraction (5.2P) with the complete activating system (protein kinase, ATP, Mg^{++}, cAMP, and theophylline) enhanced activity in the subsequent lipase assay. In 12 experiments utilizing the S_{100} fraction the activation averaged 60%, ranging from 44 to 93%; in 6 experiments utilizing the 5.2P fraction, activation averaged 64%, ranging from 48 to 75%. In the absence of either the protein kinase or the nucleotide cofactor mixture, activation was totally lost or markedly reduced (Table 1).

At an added concentration of 10^{-8} M, cAMP yielded some activation beyond that obtained in its absence; at 10^{-7} M and at higher concentrations maximal activation was obtained both in the S_{100} fraction and in the 5.2P fraction (Tables 2 and 3). In the presence of protein kinase, addition of cAMP alone or of ATP alone yielded a small but significant activation (Tables 2 and 3). However, when both were present the activation was increased 2- to 3-fold. Endogenous

TABLE 1. *Activation of partially purified lipase preparations.*

Tissue fraction	Additions	Experiment 1 Lipase activity (μeq FFA/g/hr)	Experiment 1 Percentage activation	Experiment 2 Lipase activity (μeq FFA/g/hr)	Experiment 2 Percentage activation
Soluble fraction (S_{100})	None	2.9	...	6.4	
	Complete system	5.6	93	10.2	59
	Minus kinase	3.3	14		
	Minus cofactors	2.8	0		
Acid-precipitated fraction 5.2 P)	None	2.1	...	5.0	
	Complete system	3.1	48	7.8	64
	Minus kinase	2.2	5	4.8	0
	Minus cofactors	2.2	5	5.3	4

In this and subsequent Tables, lipase activity is referred to the wet weight of original adipose tissue from which the fraction assayed was derived. Components of the complete system and conditions for incubation and assay are described under *Methods*.

TABLE 2. *Cofactor requirements for activation of lipase in the soluble supernatant fraction.*

Additions	Lipase activity (μeq FFA/g/hr)	Percentage activation
None	6.4	
Complete system with 10^{-5} M cAMP	9.2	44
Complete system with 10^{-6} M cAMP	9.8	53
Complete system with 10^{-7} M cAMP	10.2	59
Complete system with 10^{-8} M cAMP	8.6	34
Minus cAMP	7.9	23
Minus ATP	7.8	22
Minus Mg^{++}	9.2	44
Minus theophylline	10.8	69
Kinase only	7.1	11

TABLE 3. *Cofactor requirements for activation of lipase in the pH 5.2 precipitate fraction.*

Additions	Lipase activity (μeq FFA/g/hr)	Percentage activation
None	2.4	
Complete system with 10^{-5} M cAMP	4.0	67
Complete system with 10^{-6} M cAMP	3.9	63
Complete system with 10^{-7} M cAMP	3.9	63
Complete system with 10^{-8} M cAMP	3.1	29
Kinase only	2.9	21
Minus ATP	2.9	21
Minus cAMP	3.1	29
Minus Mg^{++}	3.5	46
Minus theophylline	4.2	75
Complete system but kinase heat-inactivated	2.4	0

nucleotides bound to enzyme may account for these findings. In the presence of 10^{-5} M cAMP theophylline could be omitted without loss of activation, presumably because the activation proceeds rapidly relative to the rate of phosphodiesterase hydrolysis of the added cAMP. Omission of Mg^{++} decreased but did not abolish activation. Heat-inactivated protein kinase was without effect (Table 3).

When intact fat pads are incubated with catecholamines, lipase activity in the unfractionated total homogenate[17] and in the S_{100} fraction is enhanced[11] although the magnitude of the effect is not as great as the enhancement of FFA release from the intact tissue. The data in Table 4 show that whereas the S_{100} fraction

TABLE 4. *Lipase activation in the S_{100} fraction from control and epinephrine-tested fat pads.**

Source of S_{100} fraction	Additions	Relative lipase activity†	Significance of paired differences‡
Fat pads preincubated without epinephrine	None	100	
	Cofactors only	113 ± 8.1	$P = 0.05$
	Kinase + cofactors	160 ± 13.7	$P < 0.001$
Fat pads preincubated with epinephrine	None	100	
	Cofactors only	116 ± 19.1	N.S.
	Kinase + cofactors	110 ± 7.8	N.S.

* Four pairs of fat pads were individually incubated for 4 hr in Krebs-Ringer bicarbonate buffer containing 4% bovine serum albumin. One of each pair was exposed to 5×10^{-5} M epinephrine during the last 30 min of incubation prior to homogenization.

† Lipolytic activity in the absence of kinase and cofactors was set arbitrarily equal to 100. Absolute values ranged from 4.0 to 5.5 μeq of FFA released per g. wet wt tissue represented in S_{100} fraction.

‡ Significance of differences evaluated from variance of paired differences from activity in homogenate with no additions.

derived from control fat pads showed distinct kinase-dependent lipase activation (+50 to +70%), there was no significant activation (0 to +17%) in the same fraction derived from the paired fat pads exposed to epinephrine prior to homogenization. Before protein kinase activation the total lipase activity in the S_{100} fraction from epinephrine-treated fat pads in this experiment was no greater than that in the control fractions. This suggests differential partitioning of activated lipase during homogenization and preparation of the S_{100} fraction. We have shown that the ionic composition of the homogenizing medium can importantly influence this partitioning.[11] In any case, the results shown in Table 4 suggest that little or no activatable lipase (inactive form) remained after hormone-treatment of the intact tissue.

Discussion. The present results provide the first evidence for a protein kinase step linking hormone-stimulated cAMP formation to activation of hormone-sensitive triglyceride lipase in adipose tissue. Optimal activation depended on the addition of ATP and cAMP along with protein kinase, compatible with a protein-phosphorylating activity analogous to that catalyzed by this enzyme in muscle. The physiological relevance of the demonstrated activation is supported by a number of points. First, adipose tissue has been shown by Corbin and Krebs[18] to contain a protein kinase‡. Second, there was no activation of lipase in supernatant fractions prepared from fat pads previously incubated with epinephrine. Presumably, the bulk of the hormone-sensitive lipase in these tissues had already been converted to the active form. Third, activation was significant and consistent only in preparations from fat pads in which lipolytic activity had been allowed to decline by incubating them for several hours in the absence of hormones prior to homogenization; demonstration of enhanced lipase activity in crude homogenates also depends on such prior incubation of the intact fat pads.[6,17] Finally, the concentrations of cAMP that stimulated activation in the purified fractions in these experiments were in the same range as, or lower than, the levels found in hormone-treated adipose tissue.[2]

Protein kinase from another tissue (muscle) and species (rabbit) was used because of its ready availability in purified form and because of the relative lack of substrate specificity of this enzyme.[13] As mentioned above, a single protein

kinase preparation in muscle is active both in the glycogen phosphorylase system and the glycogen synthetase system of that tissue.[14] Moreover, the preparations of protein kinase that phosphorylate phosphorylase *b* kinase also phosphorylate casein and protamine,[13] although these may not be their preferred or natural substrates. Whether the protein kinase is directly phosphorylating lipase (in analogy with the glycogen synthetase system[19]) or whether it is phosphorylating and activating a mediating enzyme, in analogy with its action in the glycogen phosphorylase system,[13] remains to be determined. In one experiment lipase purification was carried on to the preparative ultracentrifugal flotation step, which yields a 15- to 30-fold purification ($d < 1.12$ fraction).[11] Addition of kinase and cofactors gave 93% activation while addition of cAMP and ATP without kinase had essentially no effect. Also, when this purified enzyme was activated in the presence of [γ-^{32}P]ATP, radioactivity in *O*-phosphate residues of the enzyme protein was demonstrable. These observations may be considered as evidence against the participation of a second activating enzyme. However, experience in other systems cautions against such an interpretation at this time. Thus, the kinase that phosphorylates pyruvate dehydrogenase (converting it to its *in*active form) does not dissociate from the enzyme during purification but instead appears to be an integral part of an organized complex.[20–22] Furthermore, the results reported here indicate that both the inactive (or, at least, *less* active) and the active form of hormone-sensitive lipase are both retained to some extent in even the purest fractions tested. Indeed, the large size of the pure, ultracentrifugally homogenous lipase ($S_{20,w} = 32$)[12] is compatible with its representing a multienzyme complex. Thus, the possibility that additional enzymatic steps intervene between the action of the added protein kinase and the activation of lipase is not ruled out.

Activation of lipase dependent on ATP and cAMP in a cell-free system was reported by Rizack.[6] This activation, however, was obtained (in the presence of 2×10^{-5} M ATP) only at a rather critical cAMP concentration, optimal at about 2×10^{-6} M and dropping off sharply on either side, with no activation at concentrations above 3×10^{-5} M. ATP concentration was also critical, significant activation being observed only at values between 1 and 3×10^{-5} M. In the present studies cAMP effects were obtained at added concentrations as low as 10^{-8} M, and activation was obtained even at 10^{-4} M. Rizack was able to substitute high concentrations of calcium ion for cAMP and obtain comparable activation; in the present studies EDTA or EGTA was present throughout, so that a role for calcium ion is unlikely. Tsai and Vaughan have also recently reported activation of lipolytic activity in a partially purified lipase fraction in the presence of 3×10^{-5} M cAMP and 3×10^{-4} M ATP.[7] As noted, in the present studies addition of these nucleotides alone at similar concentrations gave little or no lipase activation in several experiments in which addition of nucleotides plus kinase caused 50–90% activation. The relationship between the findings of Rizack and of Tsai and Vaughan and those reported above is difficult to assess, although it is possible that the activation reported by them depended upon a cAMP stimulation of endogenous protein kinase.

Abbreviations: cAMP, cyclic adenosine 3′:5′-monophosphate; FFA, free fatty acids; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N′*-tetraacetic acid.

* This investigation was supported by PHS Research Grants HE-12373 and HE-12572, and by Dr. Huttunen's PHS International Postdoctoral Research Fellowship (FO5-TW-1386).

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‡ After these studies were completed we learned that Corbin and Krebs (personal communication) have independently observed lipase activation by rabbit muscle protein kinase in unfractionated homogenates of rat adipose tissue using the rate of release of FFA from endogenous glycerides for assay purposes.

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