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CALMODULIN-ACTIVATED PROTEIN KINASE ACTIVITY OF ADIPOCYTE MICROSOMES

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Summary. A calmodulin-activated phosphorylation activity was identified in microsomal (endoplasmic reticulum) preparations from rat adipocytes. Activity was not detected in mitochondrial or plasma membrane fractions. Although the phosphorylation of several proteins was enhanced by addition of calmodulin, the major calmodulin-sensitive protein had a molecular weight of 54,000. A series of experiments were conducted to determine if the microsomal phosphorylation was either calmodulin-containing phosphorylase kinase or calmodulin-dependent myosin light chain kinase. The phosphorylation of the 54,000 Dalton band in microsomal preparations was 1) not significantly reduced by potential competing protein substrates, e.g. actomyosin or phosphorylase b, 2) nearly equally well phosphorlyated at pH 8.6 or pH 7.0, unlike actomyosin or phosphorylase b, and 3) not increased by addition of phosphorylase kinase or myosin light chain kinase. The results demonstrate that this microsomal calmodulin-activated phosphorylation is catalysed by a protein kinase distinct from phosphorylase kinase or myosin light chain kinase.

Calmodulin was first described by Cheung (1) and Kakiuchi and Yamozaki (2) as an activator of 3', 5'-cyclic nucleotide phosphodiesterase. Subsequent studies have shown that calmodulin is a protein of 17,000 Daltons (3) which must bind calcium to produce activation, with a dissociation constant near 1 μM (4,5,6,7). In addition to cyclic nucleotide phosphodiesterase, many other enzymes are activated by calmodulin, including erythrocyte plasma membrane Ca^{2+}-Mg^{2+}-ATPase (8,9), brain adenylate cyclase (10), Ca^{2+}-ATPase of sarcoplasmic reticulum (11), and several protein kinases (12).

The calmodulin-activated protein kinases are emerging as an important class of cAMP-independent protein kinases. Calmodulin has recently been shown to be a tightly bound subunit of phosphorylase kinase (13) and appears to mediate the calcium sensitivity of this enzyme. Myosin light chain kinase from several tissues is activated by calmodulin in the presence of calcium (14,15,16,17). Other tissues have been reported to contain calmodulin-sensitive protein kinase activity, but these activities are as yet poorly

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characterized. A sedimentable protein kinase activity activated by calmodulin has been reported to be present in a variety of tissues (18). Sarcoplasmic reticulum is reported to contain a calmodulin-sensitive protein kinase which increases calcium transport (19) and Ca^{2+}-ATPase activity (20). Glycogen synthase is reported to be phosphorylated by calmodulin-stimulated protein kinase activity (21,22), which may be phosphorylase kinase (23,24).

Previous work in this laboratory established the presence of calmodulin in adipocyte preparations, and characterized the calcium-dependent activation of 3', 5'-cyclic AMP phosphodiesterase by calmodulin in this tissue (25). The present report describes a calmodulin-sensitive protein kinase activity in adipocyte microsomal fraction (endoplasmic reticulum) which is distinct from phosphorylase kinase and myosin light chain kinase.

Materials and Methods. Male Sprague-Dawley rats, weighing 120 g, were obtained from Eldridge Animal Farms, St. Louis, MO at least 24 hours before use. Carrier free [^{32}P]-phosphate and Protosol were purchased from New England Nuclear Corp. Phosphoylase b (muscle), phosphorylase kinase (muscle) and disodium ATP were from Sigma. Myosin light-chain kinase (Turkey gizzard) was the generous gift of Dr. Robert Adelstein, NIH. Actomyosin was prepared from outdated platelets by repeated precipitation-dissolution (16). Calmodulin was prepared from rat brain by a method utilizing fluphenazine agarose chromatography (26). An enzymatic method (27) was employed to make ATP labelled at the terminal phosphate with ^{32}P, which was diluted with non-labelled ATP to specific activities of 8-40 mCi/mmole.

Adipocytes were prepared by the method of Rodbell (28). Adipocyte subcellular fractions were prepared by the method of McKeel and Jarett with minor modifications (29). Protein kinase activity was assayed by measurement of radioactivity incorporated into specific protein bands following sodium dodecyl sulfate (SDS) electrophoresis (18). Incubations of 100 µL volume contained 80-200 µg protein, 50 mM Pipes-NaOH pH 7.0 or 50 mM Tris-HCl pH 8.6, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.4 mM EDTA, 0.1 mM dithiothreitol, and 5.0 µM [^{32}P]-ATP. Where indicated, incubations contained 20 µg phosphorylase b (muscle) or 15 µg actomyosin (platelet). After a 5 second incubation at 30^{0}, the reactions were stopped with 50 µL of 9% SDS - 6% 2-mercaptoethanol followed by immersion in a boiling water bath for two minutes. Aliquots of 50 µL were subjected to SDS electrophoresis(30); gels were stained for protein and dried. After autoradiography, protein bands were cut from the gel, hydrated with 0.5 ml H_2O for 30 minutes, heated at 50^{0}C with 2.0 ml Protosol for 3 hours, and the eluted radioactivity counted by liquid scintillation. Protein was determined by the method of Lowry et al. (31). All results are expressed as one standard error of the mean, derived from at least three separate experiments.

Results. When a total sedimentable fraction was prepared from adipocytes by the method of Schulman and Greengard (18) (prepared in Tris-EDTA buffer) and phosphorylation of proteins investigated using SDS gel electrophoresis/auto-

radiography (30), neither overall phosphorylation nor phosphorylation of particular protein bands were enhanced by addition of calcium and calmodulin. However, subfractionation of adipocyte membranes (in 10 mM Tris-HCl pH 7.5, 250 mM sucrose, and 1.0 mM EDTA) revealed calmodulin-stimulatable phosphorylation in the microsomal fraction, which is known to be largely endoplasmic reticulum (32). Of the several bands with increased phosphorylation, a band corresponding to a molecular weight of 54,000 (± 500) was most affected by calmodulin (Figure 1). Addition of either calcium or calmodulin alone resulted in no increase in phosphorylation, but simultaneous addition resulted in a 3.5-fold increase in phosphorylation of the 54,000 Dalton band in this experiment (Figure 1). Plasma membrane and mitochondrial subfractions contained no detectable calmodulin-stimulated phosphorylation activity.

Phosphorylase kinase (13) and myosin light chain kinase (14,15,16,17) are calmodulin-activated protein kinases. The possibility that the protein phosphorylation activity found in adipocyte microsomes was due to one of these enzymes was investigated in several experiments. When exogenous phosphorylase b or actomyosin (containing myosin light chain) was added to microsomal phosphorylation assays, both proteins were phosphorylated but to a lesser degree than the 54,000 Dalton band. Also, enhancement of phosphorylation with calmodulin (over basal phosphorylation) was 360% (± 110%) for the 54,000 Dalton band, but only 90% (± 20%) for either phosphorylase b or the light chain of myosin. The significant differences in activation of phosphorylation by calmodulin suggest that the 54,000 Dalton band was phosphorylated by a protein kinase different from the kinase(s) responsible for phosphorylase b or actomyosin phosphorylation.

If the microsomal calmodulin-activated phosphorylation was catalysed by phosphorylase kinase or myosin light chain kinase, then addition of exogenous protein substrate (phosphorylase b or actomyosin respectively) would be expected to reduce phosphorylation of the 54,000 Dalton band as the result of competition of protein substrates. In three separate experiments the addition

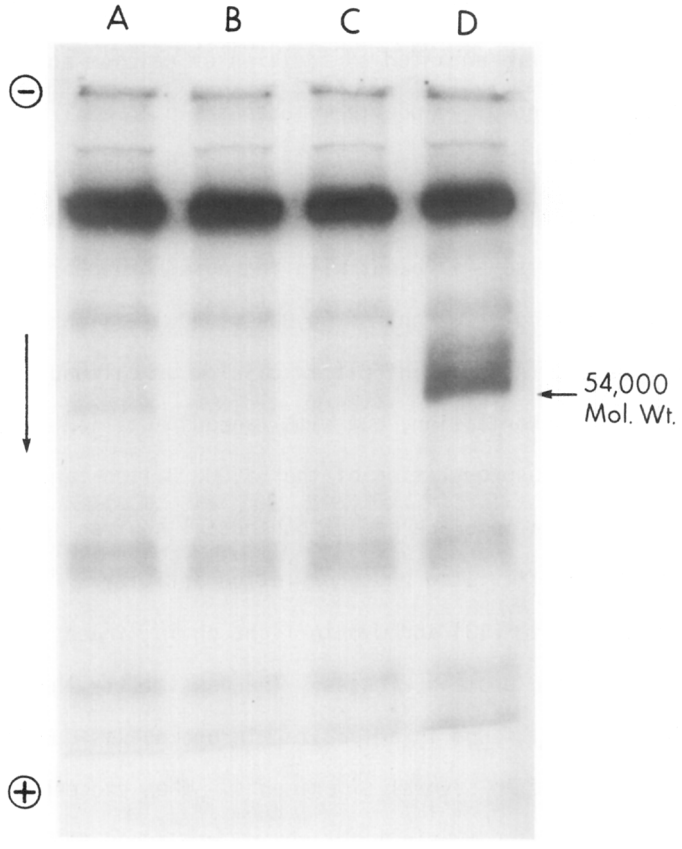

Figure 1. Calmodulin and calcium dependent phosphorylation of adipocyte microsomal (endoplasmic reticulum) proteins. This autoradiograph shows the SDS electrophoresis pattern following labelling with [^{32}P]-ATP at the incubation conditions described in methods. Slot A; control incubation without addition of calcium or calmodulin. Slot B; 5 µL of 20 mM $CaCl_2$ was added to produce a 300 µM free calcium concentration. Slot C; 0.7 µg calmodulin was added. Slot D; 0.7 µg calmodulin and 300 µM free calcium were added. Phosphorylation of a protein band of 54,000 Daltons was greatly increased by simultaneous presence of calcium and calmodulin (slot D) but not by either agent alone (slots B and C, respectively).

of phosphorylase b increased phosphorylation 1% (± 7%): addition of actomyosin reduced phosphorylation of the 54,000 Dalton band by 17% (± 10%). The small differences in 54,000 Dalton band phosphorylation in these experiments were

Table I

Effect of pH on Phosphorylation of the 54,000 Dalton Band, Phosphorylase b, and Myosin Light Chain.

Phosphorylated Protein	Ratio of Phosphorylation at pH 8.6 to pH 7.0 [a]		
	Kinase Added		
	None	Phosphorylase Kinase [b]	Myosin Light Chain Kinase [c]
54,000 Dalton Band	1.1 (± 0.1)[d]	1.1 (± 0.1)	1.0 (± 0.1)
Phosphorylase b	1.4 (± 0.2)	1.7 (± 0.1)	
Myosin Light Chain	1.4 (± 0.1)		1.4 (± 0.2)

[a] Assays were performed as described in "Methods". Each incubation contained 5 µL of 15 mM calcium to produce a 140 µM free calcium concentration, 0.7-1.0 µg of homogenous calmodulin, and either 20 µg muscle phosphorylase b or 15 µg platelet actomyosin. [b,c] All conditions the same except the incubations contained either 2.7 µg muscle phosphorylase kinase or 1.9 µg turkey gizzard myosin light chain kinase. [d] The ratios were calculated by dividing the counts per minute incorporated at pH 8.6 by counts per minute incorporated at pH 7.0. The values in parentheses are one standard error of the mean, calculated from a number of separate experiments ranging from three to eight for each mean value.

less than expected if myosin light chain kinase of phosphorylase kinase were catalysing the microsomal calmodulin-sensitive phosphorylation (23).

A series of experiments compared the phosphorylation of the 54,000 Dalton band, light chain of myosin, and phosphorylase b at pH 8.6 to that at pH 7.0. The phosphorylation of the 54,000 Dalton band was similar at either pH, yielding a pH 8.6/7.0 ratio of 1.1 (Table I). Phosphorylase b and the light chain of myosin were phosphorylated with pH 8.6/7.0 ratios of 1.4. Addition of phosphorylase kinase increased the ratio of phosphorylase to 1.7, while addition of myosin light chain kinase had no effect on the pH ratio of phosphorylation of myosin light chain. Addition of these exogenous kinases had no significant effect on the pH ratio of the 54,000 Dalton band. The results indicate that the different protein kinases, with varying pH optima, are responsible for the phosphorylation of phosphorylase b, myosin light chain, and the 54,000 Dalton band.

If the observed calmodulin-activated phosphorylation of adipocyte microsomal proteins was catalysed by endogenous phosphorylase kinase or myosin

Table II
Effects of Exogenous Kinases

Phosphorylated Protein	Percent of Phosphorylation [a]		
		Added Kinase	
	None	Phosphorylase Kinase [b]	Myosin Light Chain Kinases [c]
54,000 Dalton Band	100% [d]	102% (± 3%)	87% (± 2%)
Phosphorylase b	100%	219% (± 24%)	
Myosin Light Chain	100%		4960% (± 240%)

[a] Assays were performed as described in "Methods". Each incubation contained 5 µL of 15 mM calcium to produce a 140 µM free calcium concentration, 0.7-1.0 µg of homogeneous calmodulin, and either 20 µg phosphorylase b or 15 µg platelet actomyosin. [b,c] Incubations contained either 2.7 µg muscle phosphorylase kinase or 1.9 µg turkey gizzard myosin light chain kinase. [d] The counts per minute incorporated in the absence of exogenous protein kinase was defined as 100% incorporation, and incorporation with added exogenous kinase in each band expressed as the percentage of basal incorporation. Figures in parentheses are one standard error of the mean, calculated from three experiments for each mean value.

light chain kinase, then addition of exogenous phosphorylase kinase or myosin light chain kinase would be expected to increase phosphorylation of the 54,000 Dalton band. Addition of either kinase had negligible effects on the phosphorylation of the 54,000 Dalton band (Table II) despite the observations that added phosphorylase kinase more than doubled phosphorylation of phosphorylase b and the addition of myosin light chain kinase increased myosin light chain phosphorylation nearly 50-fold in the same incubations. The negligible effects resulting from addition of these kinases on the 54,000 Dalton band phosphorylation (Table II) despite observed significant increases in phosphorylation of phosphorylase b or myosin light chain in the same incubations, indicated that endogenous phosphorylase kinase or myosin light chain kinase was not catalysing the calmodulin-sensitive phorphorylation of adipocyte microsomal proteins.

Discussion. Unlike brain (18), total particulate preparations from rat adipocytes do not contain detectable levels of calmodulin-stimulatable phosphorylation activity. However, subfractionation to produce a microsomal preparation greatly enriched in endoplasmic reticulum (32) revealed the presence of this activity, with properties consistent with a protein kinase. The largest increase

in phosphorylation due to calmodulin occurred in a band of molecular weight 54,000, which is similar to the molecular weight reported for a highly sensitive band in brain (18). Calmodulin-activated phosphorylation activity found in both tissues may be due to the same protein kinase, which is present in larger quantities in brain.

Though phosphorylase kinase (33) and myosin light chain kinase (16) appear to be cytosolic proteins, it was important to determine if the observed phosphorylation of adipocyte microsomal proteins was due to either of these calmodulin-activated protein kinases. Several experiments indicated that the protein kinases which catalyse the phosphorylation of phosphorylase b and myosin light chain (from actomyosin) had properties unique and distinct from the adipocyte microsomal kinase which phosphorylates the endogenous microsomal 54,000 Dalton band. First, phosphorylation of the 54,000 Dalton band was not significantly reduced by addition of actomyosin or phosphorylase b. Second, the 54,000 Dalton band was phosphorylated nearly equally at pH 8.6 or 7.0, unlike phosphorylase b or actomyosin. Third, exogenous phosphorylase kinase or myosin light chain kinase did not increase phosphorylation of the 54,000 Dalton band. The results demonstrate the presence of a calmodulin-activated protein kinase in adipocyte endoplasmic reticulum, which is unique from phosphorylase kinase or myosin light chain kinase.

The function of the microsomal protein kinase activity and the identity of its protein substrates, particularly the 54,000 Dalton band, remain unknown. The key to identifying these protein substrates might lie in the recognition of an enzymatic activity or other function which is sensitive to calcium in the micromolar range and is enriched in endoplasmic reticulum. Further experiments are underway to characterize the microsomal protein kinase and demonstrate physiological control of phosphorylation. Also, preliminary experiments with a membrane fraction from rat pancreatic islets have demonstrated the presence of similar phosphorylation activity in this tissue (unpublished experiments, M. Landt, J.M. McDonald, M. McDaniel, P.E. Lacy).

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