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PARALLEL STIMULATION IN ADIPOCYTES OF THE PLASMA MEMBRANE CA^{2+}-TRANSPORT/(CA^{2+} + MG^{2+})-ATPASE SYSTEM AND MITOCHONDRIAL PYRUVATE DEHYDROGENASE BY A SUPERNATANT FACTOR DERIVED FROM ISOLATED PLASMA MEMBRANES[1]

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Summary. A factor derived from the supernatant of adipocyte plasma membranes which mimics the effects of insulin on mitochondrial pyruvate dehydrogenase (Kiechle, F.L., Jarett, L., Kotagal, N., and Popp, D.A. (1981) J. Biol. Chem. 256, 2945-2951) was shown to activate a high affinity (Ca^{2+} + Mg^{2+})-ATPase and ATP-dependent Ca^{2+} transport of adipocyte plasma membranes. Fractionation of the supernatant by G25 Sephadex chromatography separated the material into four inactive and one active fraction. All of the fractions behaved identically in terms of activation of pyruvate dehydrogenase, (Ca^{2+} + Mg^{2+})- ATPase, and Ca^{2+} transport, suggesting that the active fraction responsible for stimulating mitochondrial pyruvate dehydrogenase was also responsible for stimulating the plasma membrane Ca^{2+}-dependent processes.

The nature of the effector mechanisms which are activated following the interaction of insulin with its target cells has remained elusive (1). Two recent lines of investigation show promise in aiding the understanding of these effector mechanisms. First, a low molecular weight, acid stable and water-soluble material recovered from both insulin-treated cells and more recently from isolated adipocyte plasma membranes simulated the action of insulin on mitochondrial pyruvate dehydrogenase activity in isolated rat adipocyte mitochondria (2-9). Second, the interaction of insulin with adipocytes (10) or their isolated plasma membranes (11) resulted in the inhibition of a high affinity (Ca^{2+} + Mg^{2+})-ATPase which appears to serve as the enzymatic

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basis for a high affinity, calmodulin-sensitive plasma membrane Ca^{2+} transport system (12,13).

In this communication, it is demonstrated that : 1) the supernatant derived from adipocyte plasma membranes stimulates both high affinity (Ca^{2+} + Mg^{2+})-ATPase and ATP-dependent Ca^{2+} transport activities in adipocyte plasma membranes, and 2) the effects of chromatographically separated fractions of the supernatant from adipocyte plasma membranes have similar effects on (Ca^{2+} + Mg^{2+})-ATPase activity, Ca^{2+} transport, and pyruvate dehydrogenase.

Materials and Methods. Adipocytes were prepared from the epididymal fat pads of 120 g male Sprague-Dawley rats as described previously (14). Plasma membranes and mitochondria were prepared as previously described either with (15) or without (16) EDTA in the homogenization and fractionation buffers. The supernatant derived from adipocyte plasma membranes was obtained by resuspending the plasma membranes in 50 mM phosphate buffer, pH 7.4 to a final concentration of 1-3 mg protein/ml. After incubating 30 sec at 37°C, the plasma membranes were pelleted in a Beckman microfuge for 45 sec (5). The resultant supernatant was rapidly frozen at -70°C for use in the assays (50 mM phosphate buffer was used in control incubations) or was further fractionated by Sephadex G25 chromatography as described by Kiechle et al. (5). Fractions I-V as previously described (3,5) were diluted in 60 ml 0.05 M formic acid, lyophilized, and reconstituted in 1.5 ml of 0.001 M formic acid before addition to the assays. All assay controls contained an equivalent amount of material prepared by lyophilizing 60 ml of 0.05 M formic acid followed by reconstitution in 1.5 ml of 0.001 M formic acid.

(Ca^{2+} + Mg^{2+})-ATPase assays were performed with isolated adipocyte plasma membranes as previously described (13) in a standard assay buffer containing 10-20 µg protein/ml, 2 mM Tris/EGTA, 1.8 mM $CaCl_2$ (0.143 µM calculated free [Ca^{2+}]), 1 mM Na_2ATP (with tracer [^{32}P]-ATP), 20 mM NaN_3, and 12.5 mM Tris/1,4-piperazine-diethanesulfonic acid, pH 7.5, at 35°C. Ca^{2+}-stimulated activity was determined after a 30 min incubation at 37°C by subtracting values obtained with EGTA alone from those obtained with Ca^{2+} plus EGTA.

ATP-dependent Ca^{2+} transport in adipocyte plasma membranes was performed as described previously (12). The standard assay medium contained 30-40 µg protein, 200 mM sucrose, 20 mM NaN_3, 10 mM Tris/oxalate, 2 mM $MgCl_2$, 2 mM Tris/EGTA, 1.8 mM $CaCl_2$ (with tracer $^{45}CaCl_2$), and 10 mM Tris/1,4-piperazine-diethanesulfonic acid, pH 7.5, at 37°C. As in the ATPase buffer, the calculated free [Ca^{2+}] in this system is 0.143 µM. The added oxalate has no effect on this concentration because EGTA is the principal buffer for Ca^{2+}. Incubation time was 20 min at 37°C. Assays were terminated by rapid filtration and washing. ATP-dependent uptake was determined by subtracting values obtained in the absence of ATP from those obtained in the presence of ATP.

Pyruvate dehydrogenase assays were performed using mitochondria isolated from adipocytes (3). In these experiments 50 µl of each fraction obtained from Sephadex G25 fractionation of the plasma membrane supernatant material

TABLE I

Stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase by the Supernatant from Adipocyte Plasma Membranes[1]

Experiment	$(Ca^{2+} + Mg^{2+})$-ATPase Activity	
	Control	+ Supernatant
	nmol P_i/mg protein/min	
1	17.9 ± 1.1	180.7 ± 5.8
2	21.6 ± 2.7	69.8 ± 10.4
3	41.3 ± 8.8	204.9 ± 34.9
4	82.7 ± 2.9	253.9 ± 8.2
$\bar{X}$ ± SEM	40.9 ± 12.9	177.3 ± 33.7

[1]ATPase assays were performed in triplicate as described in the Methods using four different plasma membrane preparations prepared in the absence of EDTA. The supernatant (from four different preparations) was added at a concentration of 100 µl/ml. The supernatant contained no detectable $(Ca^{2+} + Mg^{2+})$-ATPase activity.

was preincubated with 150 µl containing 250-500 µg mitochondrial protein, 50 µM $MgCl_2$, and 50 µM $CaCl_2$ in 50 mM potassium phosphate buffer, pH 7.4, for 5 min at 37°C (5). The assay was initiated by the addition of 0.25 mM 1-^{14}C pyruvate (1 mCi/mM), 0.5 mM NAD, 1 mM coenzyme A, 0.1 mM cocarboxylase, and 0.1 mM dithiothreitol in 50 mM phosphate buffer, pH 7.4. After 2 min, the assay was stopped by the addition of 200 µl of 6 M H_2SO_4, and enzyme activity was quantitated by measuring the amount of $^{14}CO_2$ produced (3).

Results. The unfractionated supernatant derived from adipocyte plasma membranes markedly stimulated the high affinity $(Ca^{2+} + Mg^{2+})$-ATPase activity (Table I). In these experiments the membranes had been stored at -70°C for 0-14 days, which most likely accounted for the variable $(Ca^{2+} + Mg^{2+})$-ATPase activity in the control experiments (13). Nevertheless, the addition of the supernatant material clearly stimulated activity from three to ten-fold in each experiment. Furthermore, heating the supernatant material for 10 min at 100°C abolished the stimulatory effect on enzyme activity, indicating that the supernatant factor responsible for this stimulation was neither calmodulin (which is heat-resistant) nor inorganic ions (such as Ca^{2+} or Mg^{2+}), which would be unaffected by boiling. Heating the supernatant material for 10 min at 100°C also abolished the stimulatory effect on pyruvate dehydrogenase activity (data not shown). The ability of the supernatant material to stimu-

TABLE II

Stimulation of ATP-Dependent Ca^{2+} Transport by the Supernatant from Adipocyte Plasma Membranes[1]

Experiment	Ca^{2+} Transport	
	Control	+ Supernatant
	nmol Ca^{2+}/mg protein/10 min	
1	3.3 ± 0.2	6.6 ± 0.5
2	7.2 ± 0.1	11.0 ± 0.1
3	11.0 ± 2.6	28.1 ± 1.1
$\bar{X}$ ± SEM	7.2 ± 1.8	15.2 ± 5.4

[1] Ca^{2+} transport assays were performed in triplicate as described in the Methods. Experiment number 1 utilized plasma membranes prepared in the presence of 1 mM EDTA while Experiments number 2 and number 3 utilized different membranes prepared in the absence of EDTA. Supernatant (three different preparations) was added at a concentration of 50 µl/ml.

late the (Ca^{2+} + Mg^{2+})-ATPase was also concentration-dependent. In one experiment, 20 µl of supernatant stimulated (Ca^{2+} + Mg^{2+})-ATPase activity by 64.2% or 29.6 nmol P_i/mg/min, whereas 100 µl stimulated activity by 396% or 163.6 nmol P_i/mg/min. A similar concentration-dependence has been shown for the stimulation of pyruvate dehydrogenase by the supernatant (5).

The supernatant from adipocyte plasma membranes similarly stimulated ATP-dependent Ca^{2+} transport in adipocyte plasma membranes (Table II). In three separate experiments the percent stimulation of Ca^{2+} transport ranged from 52.7% to 155.5%, with a mean value of 102.7%. In experiment number 1 the plasma membranes were prepared in EDTA, which may account for the lower level of transport in the controls. EDTA will remove calmodulin, which has been shown to stimulate transport (12), resulting in a lower level of basal transport. As in the ATPase experiments, boiling the supernatant for 10 min completely abolished the stimulatory effect, indicating that neither calmodulin nor inorganic ions were responsible for the observed stimulation by the active component of the supernatant.

Finally, fractionation of the supernatant by Sephadex G25 chromatography yielded 5 fractions as previously described (5,6) which behaved similarly

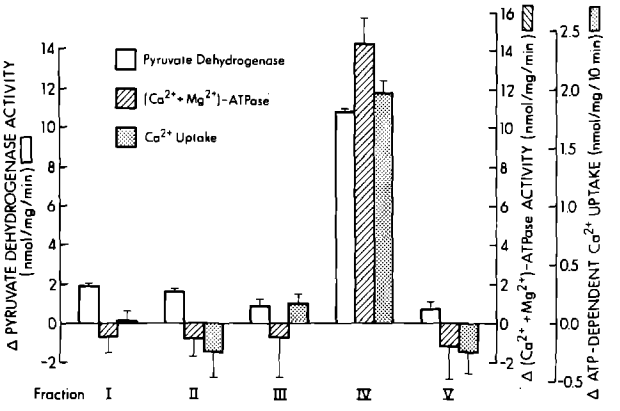

Figure 1: The effects of the fractions from Sephadex G25 chromatography of the supernatant from adipocyte plasma membranes on pyruvate dehydrogenase, $(Ca^{2+} + Mg^{2+})$-ATPase and ATP-dependent Ca^{2+} transport. The plasma membrane supernatant was prepared and fractionated by Sephadex G25 chromatography as described in "Materials and Methods". Equal aliquots of each fraction were added to all assays as outlined in "Materials and Methods". Results are expressed as the difference between the values obtained with each fraction and the control values. Each assay was performed in triplicate and the bars represent the SEM.

in three different assays: $(Ca^{2+} + Mg^{2+})$-ATPase, Ca^{2+} transport, and pyruvate dehydrogenase (Figure 1). As can be seen, the ability to stimulate all three activities was observed only in fraction IV. Fractions I, II, III, and V altered the activity of all three parameters only slightly. These data therefore suggest that the same active fraction of the supernatant from adipocyte plasma membranes which is responsible for activating pyruvate dehydrogenase is also responsible for activating $(Ca^{2+} + Mg^{2+})$-ATPase and ATP-dependent Ca^{2+} transport in adipocyte plasma membranes.

Discussion. A putative chemical mediator of insulin with an apparent molecular weight of 1000-1500 (3,5,7) has been partially purified from several sources and has been shown to mimic insulin action on several enzyme systems including glycogen synthase (7) and pyruvate dehydrogenase (8). Insulin increased the activity or quantity of the mediator from skeletal muscle (7), adipocytes (3), and adipocyte plasma membranes (5). The recent demonstration that insulin could directly inhibit a high affinity $(Ca^{2+} + Mg^{2+})$-ATPase

in adipocyte plasma membranes (10) has suggested that this enzyme and the associated Ca^{2+} transport system could play an important primary role in the mechanism of insulin action. It was therefore logical to test the effect of the putative chemical mediator of insulin action on the $(Ca^{2+} + Mg^{2+})$-ATPase/Ca^{2+} transport system in adipocyte plasma membranes.

The unchromatographed supernatant stimulates both $(Ca^{2+} + Mg^{2+})$-ATPase (Table I) and Ca^{2+} transport (Table II). Furthermore, the active fraction of the chromatographed supernatant which stimulated mitochondrial pyruvate dehydrogenase was also the fraction which stimulated both $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} transport (Figure 1). In contrast, the direct addition of insulin to isolated plasma membranes or insulin treatment of intact adipocytes prior to isolation of the plasma membranes resulted in an inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase compared to control plasma membranes (10,11).

Therefore, the plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase is apparently modulated in opposing directions by the insulin molecule itself and its putative chemical mediator. There are several possible explanations for these opposing effects. First, the binding of insulin to its receptor may release both a mediator and an antimediator (17), thereby providing its own self-regulating negative feedback system. Therefore under conditions in which direct addition of insulin to plasma membranes inhibits $(Ca^{2+} + Mg^{2+})$-ATPase activity, production of antimediator may be favored. Second, the binding of insulin to its receptor on the plasma membrane may directly inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase. The chemical mediator may overcome this inhibition, if produced in sufficient quantity, but with the plasma membrane concentration used in the $(Ca^{2+} + Mg^{2+})$-ATPase assay it is unlikely that such quantities of the mediator are produced.

It is clear that calmodulin is not the factor in the supernatant that is responsible for the stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase/Ca^{2+} transport system. First, the stimulatory activity of the supernatant on pyruvate dehydrogenase, $(Ca^{2+} + Mg^{2+})$-ATPase, and Ca^{2+} transport were all

abolished by heating at 100°C for 10 min. Calmodulin is heat-stable and is not inactivated by boiling (18). Second, the active fraction (Fraction IV, Figure 1) is a low molecular weight compound (5), whereas calmodulin has a MW of approximately 17,000 (18). However, the chemical mediator may cause activation of the $(Ca^{2+} + Mg^{2+})$- ATPase/Ca^{2+} transport system through membrane-bound calmodulin. Calmodulin has been shown to stimulate the Ca^{2+} transport system. Therefore both the inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase by insulin and/or the stimulation caused by the chemical mediator could be caused by local alterations in the plasma membrane bound calmodulin and/or other plasma membrane associated regulator molecules, which then affects the $(Ca^{2+} + Mg^{2+})$-ATPase/Ca^{2+} transport system. The chemical mediator has been shown to exert its primary regulatory effects through phosphorylation and dephosphorylation (6), and calmodulin appears to act through regulation of phosphorylation (18). The insulin-mediated regulation of the $(Ca^{2+} + Mg^{2+})$-ATPase appears to involve the phosphorylation of 110,000 dalton phosphorylated intermediate of the $(Ca^{2+} + Mg^{2+})$-ATPase (19,20). The mechanisms whereby the supernatant factor, calmodulin, and insulin regulate this phosphoprotein is unknown. However, continued investigation of these mechanisms should yield important information about the molecular mechanism of insulin action at the plasma membrane.

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