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Insulin Activation of (Na^+,K^+)-Adenosinetriphosphatase Exhibits a Temperature-Dependent Lag Time. Comparison to Activation of the Glucose Transporter†

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ABSTRACT: The time course of insulin activation of sodium and potassium ion activated adenosinetriphosphatase [(Na^+,K^+)ATPase] was studied in the rat adipocyte and was compared to activation of the glucose transporter. Under conditions in which the binding of insulin to its cell surface receptor was not rate limiting, a distinct time lag was apparent between insulin addition and stimulation of transport activity. At 37 °C, 40–50 s elapsed before an increase in Rb^+ uptake [a measure of (Na^+,K^+)ATPase transport activity] or 2-deoxyglucose uptake could be observed. This lag time increased in an identical manner for both transport processes as the temperature was lowered to 23 °C. Addition of the insulinomimetic agent hydrogen peroxide also produced a lag time similar to that for insulin before activation of Rb^+ and 2-deoxyglucose uptakes was detected. These data provide the first evidence of a discrete time lag involved during stimulation of the adipocyte (Na^+,K^+)ATPase. A model for the molecular mechanism of insulin activation of (Na^+,K^+)ATPase is presented that incorporates these results into the hypothesis of insulin mediated "translocation" of glucose transporters to the plasma membrane.

Active transport of sodium and potassium ions across the plasma membrane of fat and muscle cells is an insulin-sensitive process mediated by stimulation of sodium and potassium ion activated adenosinetriphosphatase (Resh et al., 1980; Clausen & Kohn, 1977). There are remarkable similarities in the manner that both the adipocyte (Na^+,K^+)ATPase[1] and the transport system for glucose respond to insulin. Insulin activation of both transport proteins occurs rapidly (Resh et al., 1980; Clausen & Kohn, 1977), with an identical dose–response relationship (Resh, 1982a), and is observed with several insulinomimetic agents (Resh, 1982c). Moreover, during in vitro adipocyte differentiation, the insulin responsiveness of the Na^+ pump and the glucose transporter is coordinately expressed and regulated (Resh, 1982a). These observations support the proposal that insulin's interaction with its receptor generates a common signal which activates two different membrane transport proteins.

The key to understanding the molecular mechanism of insulin action rests on elucidation of the events which "couple" binding to the receptor to activation of the biological responses. Insulin activation of glucose transport is a time-, temperature-, and energy-dependent process (Kono, 1982), which, even at maximally effective insulin concentrations, exhibits an absolute lag time before increased transport is observed (Cirialdi & Olefsky, 1979; Haring et al., 1981). Recent evidence from the laboratories of Cushman (Karnieli et al., 1981) and Kono (Kono et al., 1982) suggests that part of this process involves an insulin-mediated "translocation" of glucose transporters from an intracellular pool to the plasma membrane. In contrast, data from this laboratory indicate that no such translocation of (Na^+,K^+)ATPases is effected by insulin treatment of adipocytes (Resh, 1982b). However, it is not known whether insulin activation of (Na^+,K^+)ATPase also involves a lag time before Rb^+ transport activity is stimulated. In this paper, the kinetics of insulin activation of (Na^+,K^+)ATPase in the rat adipocyte are investigated. The studies reported herein demonstrate that a temperature-dependent lag time occurs during activation of (Na^+,K^+)ATPase, and these results

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[1] Abbreviation: (Na^+,K^+)ATPase, sodium and potassium ion activated adenosinetriphosphatase.

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are considered in the context of the previously proposed (Karnieli et al., 1981; Kono et al., 1982) translocation hypothesis.

Experimental Procedures

Simultaneous uptake of $^{86}Rb^+$ and 2-deoxy[^{3}H]glucose into rat adipocytes was monitored as described previously (Resh et al., 1980). The adipocyte suspension was made up to a 30% packed cell volume in Krebs–Ringer phosphate buffer, pH 7.4, containing 4% (w/v) bovine serum albumin. Following a 20-min preincubation at the indicated temperature, the transport assay was initiated by the addition of 0.1 mM 2-deoxyglucose, 3.6 μCi/mL 2-deoxy[^{3}H]glucose, and 3 μCi/mL ^{86}RbCl. Duplicate 100-μL aliquots were withdrawn at the indicated times and processed as described (Resh et al., 1980).

The experimental data were fitted to a straight line by means of linear regression by the method of least squares. The intersection of the lines describing the data from control and insulin-treated samples was determined algebraically. The difference between the time point of intersection and the time of insulin addition is defined as the lag time.

It should be noted that Rb^+ and 2-deoxyglucose uptake values represent accumulation of radioisotopic substrate at each time point, rather than the actual rate constant, and therefore measure the additive effects of multiple energy- and temperature-dependent steps including transport.

Results and Discussion

The transport activities of both (Na^+,K^+)ATPase and the glucose transporter can be measured and directly compared by monitoring the simultaneous uptake of $^{86}Rb^+$ and 2-deoxy[^{3}H]glucose into rat adipocytes (Resh, 1982a). At 30 °C, there is a distinct lag time of 110–130 s following addition of a supramaximal concentration of insulin (20 nM) until increases in the accumulation of both $^{86}Rb^+$ and 2-deoxy-[^{3}H]glucose are observed (Figure 1A,B). The length of this lag time is temperature dependent: at 37 °C, it is shortened to 40–50 s, whereas at 20 °C, nearly 180 s elapses before Rb^+ and 2-deoxyglucose transport increases (Figure 1C). These data agree extremely well with the results obtained for glucose transport by Cirialdi & Olefsky (1979) and Haring et al. (1981). More importantly, the results presented in Figure 1 provide the first demonstration of a discrete lag phase during insulin activation of (Na^+,K^+)ATPase, which is indistinguishable in its time and temperature dependence from that of the glucose transporter.

It is well established that maximal activation of glucose transport (Kono & Barham, 1971) and Rb^+ uptake (Resh et al., 1980; Resh, 1982a) occurs when only a small percentage of the available receptors are occupied by insulin. At 20 nM insulin, a maximally effective amount of insulin is bound to the fat cell in less than 10 s at temperatures between 15 and 37 °C (Cirialdi & Olefsky, 1979, 1982); i.e., after 10 s, receptor binding is not a rate-limiting step for insulin action. Furthermore, delays in the ability to measure rapid changes in glucose (Cirialdi & Olefsky, 1979) and Rb^+ transport[2] cannot account for the lag observed in Figure 1. Therefore, it is reasonable to conclude that the lag time represents the coupling step(s) between binding and activation of transport

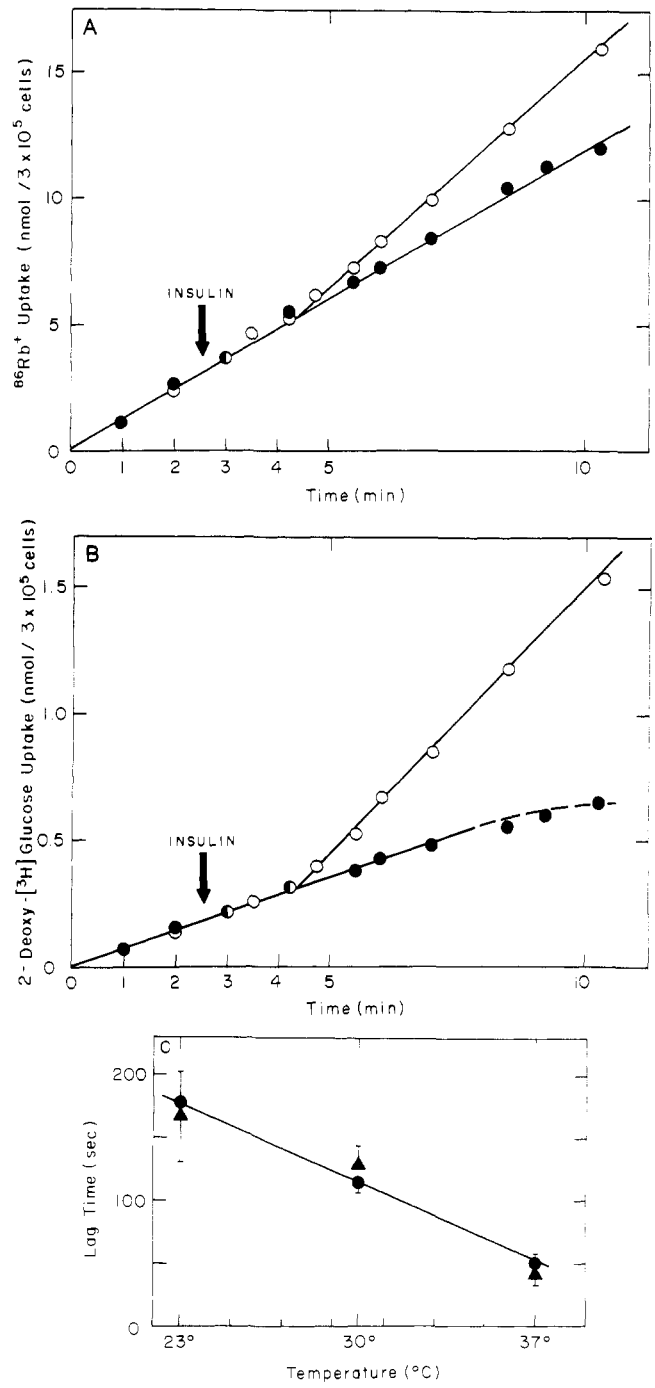

FIGURE 1: Time course of insulin activation of (Na^+,K^+)ATPase and the glucose transporter. Two tubes of fat cells were incubated, and transport activity was monitored at 30 °C as described under Experimental Procedures. At time 2.5 min, one tube received 20 nM porcine insulin (100 ng/mL) (○); the control tube received an equivalent volume of buffer (●). All data points represent the average of duplicate determinations. The size of the symbols represents the standard deviation of the data. The data were fit to straight lines by linear regression (correlation coefficient $r = 0.99$). (A) Rb^+ uptake; (B) 2-deoxyglucose uptake. In this experiment, the calculated lag times were 125 s for Rb^+ and 115 s for 2-deoxyglucose. (C) Temperature dependence of the lag time. Experiments identical with those depicted in (A) and (B) were performed in triplicate at 23, 30, and 37 °C. The lag times for stimulation of Rb^+ (▲) and 2-deoxyglucose (●) uptakes were then determined. The error bars represent the standard deviation of the data. There was no statistically significant difference between the data obtained for Rb^+ and 2-deoxyglucose ($p > 0.10$, paired Student's t test).

and that the rate-determining steps for activation of the glucose transporter and (Na^+,K^+)ATPase are kinetically indistinguishable.

[2] Rapid alterations of the extracellular K^+ concentration produced concomitant corresponding changes in $^{86}Rb^+$ uptake within 15 s (the earliest time point measured) at 20 °C (M. D. Resh, unpublished observations). Thus the response time of the transport system, as well as the time required to separate cells from the medium (<2 s), is much less than the observed lag time.

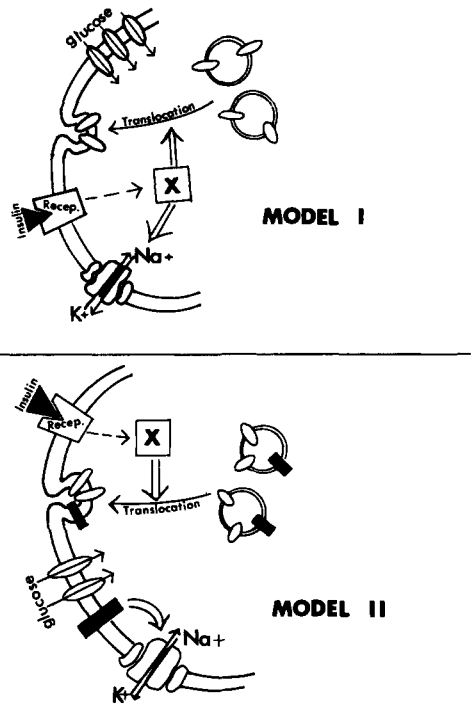

FIGURE 2: Two models for insulin activation of (Na^+,K^+)ATPase. The "translocation" model of Karnieli et al. (1981) has been modified to include stimulation of the Na^+ pump. Model I: Direct activation. Insulin binding to its cell-surface receptor generates a signal ("X") that stimulates translocation and fusion of glucose transporter containing vesicles to the plasma membrane. Factor "X" also activates (Na^+,K^+)ATPase. Model II: Activation following translocation. Insulin binding to its cell-surface receptor generates a signal ("X") that stimulates translocation of vesicles containing glucose transporter *and* an additional protein (solid rectangle). Following fusion with the plasma membrane, this other protein (rectangle) functions to activate (Na^+,K^+)ATPase. In both models, activation of the Na^+ pump occurs only in an intact cell, and insulin treatment does not change the number of Na^+ pumps (Resh, 1982b).

Hydrogen peroxide is a compound that mimics insulin stimulation of both glucose (Czech, 1976) and Rb^+ (Resh, 1982c) uptake, even in cells in which the cell-surface insulin receptors have been destroyed by trypsin treatment[3] (Kono et al., 1982). Activation of transport by 10 mM H_2O_2 gave results similar to those depicted in Figure 1A,B; at 30 °C there was a lag time of approximately 140 s before increased accumulation of both Rb^+ and 2-deoxyglucose was observed (data not shown). Since H_2O_2 acts at a step distal to the insulin binding event, this experiment supports the contention that interaction with the receptor is not rate limiting under these conditions.

There are several conceivable interpretations of the results presented in this paper. It is certainly possible that the similarity in lag times and temperature coefficients observed is simply coincidental. However, in light of the ample evidence presented (Resh et al., 1980; Resh, 1982a–c) for the similarities between activation of glucose and Rb^+ transport, it is more reasonable to incorporate these data into the "translocation" hypothesis (Karnieli et al., 1981; Kono et al., 1982) in order to describe a molecular mechanism for insulin activation of (Na^+,K^+)ATPase. If we assume that a common signal activates the glucose transporter and (Na^+,K^+)ATPase (Resh et al., 1980; Resh, 1982a,c) and that no change in the number of Na^+ pumps occurs (Resh, 1982b), then two limiting types of mechanisms are conceivable. It is possible that the signal which triggers vesicle fusion also *directly* activates (Na^+,K^+)ATPase. In this case, "translocation" of intracellular vesicles is not a prerequisite for stimulation of the Na^+ pump, and the observed lag phase would represent the time required to generate a sufficient quantity of signal "X". Alternatively, the vesicles that contain glucose transporters could also contain another protein, which, following fusion with the plasma membrane, activates the Na^+ pump. Under these circumstances, part or all of the lag phase could represent the translocation process. These two models are illustrated schematically in Figure 2. It should be emphasized that "translocation" is used here as a descriptive term for the phenomenon that causes activation of latent membrane proteins. Since definitive morphological evidence demonstrating vesicle translocation is not yet available, this mechanism must still be treated as a hypothesis.

It is not possible to definitively distinguish between these two models unless additional assumptions are made. By analogy with exocytosis, it is probable that vesicle translocation is a temperature- and energy-dependent process, as has been noted by Kono et al. (1981) and Ezaki & Kono (1982). If one assumes that the signal which triggers this event is rapidly generated, then a mechanism analogous to model II could be invoked to explain the time- and temperature-dependent stimulation of the Na^+ pump.[4] The lag time observed with H_2O_2 is also consistent with the translocation mechanism of model II, if it is assumed that peroxide mimics the action of the signal "X". It is also possible that additional time-requiring steps are involved subsequent to vesicle fusion, as has been suggested by Karnieli et al. (1981). In conclusion, the data presented in this paper demonstrate that a common time- and temperature-dependent step or steps are involved during insulin activation of (Na^+,K^+)ATPase and the glucose transporter. It will obviously be necessary to elucidate the biochemical nature of these coupling events in order to ultimately understand the molecular mechanism of insulin action.

Acknowledgments

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Registry No. Insulin, 9004-10-8; ATPase, 9000-83-3; 2-deoxyglucose, 154-17-6; rubidium, 7440-17-7.

References

Cirialdi, T. P., & Olefsky, J. M. (1979) *Arch. Biochem. Biophys. 193*, 221–231.

Cirialdi, T. P., & Olefsky, J. M. (1982) *Biochemistry 21*, 3475–3480.

Clausen, T., & Kohn, P. G. (1977) *J. Physiol. (London) 265*, 19–42.

Czech, M. P. (1976) *J. Biol. Chem. 254*, 1164–1170.

Ezaki, O., & Kono, T. (1982) *J. Biol. Chem. 257*, 14306–14310.

Haring, H. U., Biermann, E., & Kemmler, W. (1981) *Am. J. Physiol. 240*, E556–E565.

Karnieli, E., Zarnowski, M. J., Hissin, P. J., Simpson, I. A., Salans, L. B., & Cushman, S. W. (1981) *J. Biol. Chem. 256*, 4772–4777.

Kono, T. (1982) *Biochem. Soc. Trans. 10*, 9–10.

Kono, T., & Barham, F. W. (1971) *J. Biol. Chem. 246*, 6210–6216.

[3] M. D. Resh, unpublished results.

[4] It is not possible to determine if energy is independently required for insulin activation of the Na^+ pump since the basal activity of this enzyme is energy dependent.

Kono, T., Suzuki, K., Dansey, L. E., Robinson, F. W., & Blevins, T. L. (1981) *J. Biol. Chem. 256*, 6400–6407.
Kono, T., Robinson, F. W., Blevins, T. L., & Ezaki, O. (1982) *J. Biol. Chem. 257*, 10942–10947.
Resh, M. D. (1982a) *J. Biol. Chem. 257*, 6978–6986.
Resh, M. D. (1982b) *J. Biol. Chem. 257*, 11946–11952.
Resh, M. D. (1982c) Ph.D. Thesis, Harvard University, Cambridge, MA.
Resh, M. D., Nemenoff, R. A., & Guidotti, G. (1980) *J. Biol. Chem. 255*, 10938–10945.

3-Hydroxy-3-methylglutaryl-CoA Reductase: Solubilization in the Presence of Proteolytic Inhibitors, Partial Purification, and Reversible Phosphorylation–Dephosphorylation†

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ABSTRACT: A growing body of evidence indicates that 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34, reductase) is degraded by proteolytic enzymes during solubilization by traditional freeze–thaw techniques. We have solubilized reductase in an active, stable form with nonionic detergents [Lubrol WX or poly(oxyethylene) ether type W-1]. Solubilization proceeded in high (>70%) yield in the presence of the proteolytic inhibitors leupeptin, phenylmethanesulfonyl fluoride, and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid and was independent of prior freeze–thawing of the microsomes. We have purified detergent-solubilized reductase 40-fold in high yield by means of sucrose density gradient centrifugation and dye–ligand chromatography. Detergent-solubilized reductase is heat labile, unlike reductase solubilized by the freeze–thaw method. Detergent-solubilized reductase can be inactivated up to 90% by use of reductase kinase. This inactivation requires both adenosine 5′-triphosphate and adenosine 5′-diphosphate, as has been previously observed for both microsomal and freeze–thaw solubilized reductase. Inactivation is reversed by subsequent treatment with a phosphoprotein phosphatase.

3-Hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34, reductase[1]), the rate-limiting enzyme in mammalian polyisoprenoid biosynthesis (Rodwell et al., 1976), traditionally has been solubilized by procedures that subject rat liver microsomes to freeze–thawing (Brown et al., 1973; Heller & Gould, 1973). Reductase solubilized in this manner has been purified to homogeneity, and its properties have been studied in several laboratories [for a review, see Kleinsek et al. (1981)]. Ness et al. (1981) observed, however, that inclusion of the sulfhydryl protease inhibitor leupeptin or careful removal of lysosomes from the microsomal fraction prior to freeze–thawing blocked subsequent freeze–thaw solubilization. Moreover, addition of lysosomes to lysosome-depleted microsomes restored the susceptibility of reductase to solubilization by freeze–thawing. They therefore concluded that freeze–thaw solubilization relies upon cleavage of reductase by lysosomal proteases. This observation has been confirmed by Chin et al. (1982a). Using immunodetection techniques, they observed that the subunit relative molecular mass of reductase from UT-1 Chinese hamster ovary cells extracted in the presence of leupeptin was larger (62 000) than that observed when leupeptin was omitted (50 000–55 000). They later reported that inclusion of EGTA, in addition to leupeptin, yielded a reductase subunit M_r of 90 000, a relative molecular mass that matched that from immunoprecipitates of in vitro translation products of total UT-1 cellular polyadenylated RNA (Chin et al., 1982b). We have developed a method for solubilizing reductase in high yield in the presence of inhibitors of proteolysis without prior freeze–thawing of microsomes, have purified the reductase thus solubilized 40-fold, and have studied its properties.

Materials and Methods

Materials. Purchased materials included the following: Lubrol WX, poly(oxyethylene) ether type W-1, leupeptin, PMSF, EGTA, octyl glucoside, Amberlite XAD-2, and Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO); Quick Seal centrifuge tubes (Beckman Instruments, Palo Alto, CA); phenoxyacetylcellulose (Regis Chemical Co., Morton Grove, IL); Matrex Gel Green A and ultrafiltration membranes (Amicon Corp., Danvers, MA); Biobeads SM-2 (Bio-Rad Laboratories, Richmond, CA); fluorescamine (Roche Diagnostics, Nutley, NJ); deoxycholate (Difco Laboratories, Detroit, MI). [3-^{14}C]HMG-CoA was prepared as previously described at a specific activity of 1–2 cpm/pmol (Williamson & Rodwell, 1981). Other materials were from previously listed sources (Harwood & Rodwell, 1982; Nordstrom et al., 1977).

Enzymes. Low molecular weight phosphoprotein phosphatase was purified through the ammonium sulfate fractionation as described by Nordstrom et al. (1977). Reductase kinase was purified through the Blue Sepharose step (0.44 mg of protein/mL, 0.2 picounit of microsomal reductase inactivated min^{-1} mg^{-1}) as described by Harwood (1982). Freeze–thaw solubilized reductase was purified through the heat fraction as described by Rogers et al. (1980).

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[1] Abbreviations: reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; Lubrol, Lubrol WX; PEW-1, poly(oxyethylene) ether type W-1; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HMG, 3-hydroxy-3-methylglutaric acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; NADP$^+$, nicotinamide adenine dinucleotide phosphate; CoASH, coenzyme A sulfhydrylated; M_r, relative molecular mass.

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