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10. By putting the oocytes under paraffin oil, one avoids the loss of endogenous and injected solutes that can accompany microinjection in aqueous media [D. S. Miller, Y.-T. Lau, S. B. Horowitz, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1426 (1984)]. In spite of this unphysiological extracellular fluid, amphibian oocytes remain viable in oil for at least 18 hours as judged by maintenance of normal ion, water, and adenosine triphosphate content, the ability to synthesize protein and RNA, and (in full-grown oocytes) response to progesterone [Y. T. Lau, J. K. Reynhout, S. B. Horowitz, *Dev. Biol.* **104**, 106 (1984); S. B. Horowitz and D. S. Miller, *J. Cell Biol.* **99**, 172S (1984); D. S. Miller and S. B. Horowitz, *J. Biol. Chem.* **261**, 13911 (1986); D. S. Miller, unpublished data].
11. Data presented here are for animals used during the winter, spring, and fall of 1987. In June, July, and August, *Xenopus* ovaries spontaneously enter a rapid phase of oogenesis. During the summer, stage IV oocytes generally exhibited average GTP incorporation rates exceeding 0.04 per hour. In addition, the variability among cells from the same ovary increased substantially, and biochemical responses to insulin were greatly reduced or abolished.
12. The average insulin concentration in cell water (in nanomoles per liter) can be estimated by multiplying by 4 the number of femtomoles injected (calculation assumes: 0.5-mg cell mass, 50% water content, and unit cell density). However, my initial microinjection experiments with [^{125}I]insulin show that within 2 to 3 hours most of the injected hormone had been degraded; that is, it was not longer precipitable with trichloroacetic acid (TCA). Insulin degradation complicates attempts to relate the dose levels used here to receptor-binding characteristics, since neither the identity nor the local concentration of the active ligand is known.
13. Under oil, an oocyte is punctured at the animal pole with a 29-gauge hypodermic needle, and the nucleus is extruded through the hole by gently squeezing the cell at the equator. Nuclei isolated in this manner are essentially free of cytoplasmic contamination, as judged by light and electron microscopy and by the absence of yolk proteins in two-dimensional polyacrylamide gels. A comparison of two-dimensional gels from oocyte nuclei isolated under oil with those from nuclei isolated by cryomicrodissection shows that nuclear proteins are not lost during isolation in oil. Moreover, oocyte nuclei isolated in oil retain the ability to synthesize RNA from labeled GTP or uridine triphosphate added in microdrops. Synthesis continues for many hours and is sensitive to inhibition by actinomycin and Sarkosyl [S. B. Horowitz *et al.*, *J. Cell Biol.* **101**, 211a (1985); S. B. Horowitz *et al.*, in preparation]. Use of paraffin oil as an isolation medium avoids the following problems associated with conventional water-based procedures: (i) physical damage caused by tissue homogenization and (ii) massive loss of endogenous proteins, cofactors, ions, and metabolites through the porous nuclear envelope to the aqueous medium [P. L. Paine, C. F. Austerberry, L. J. Desjarlais, S. B. Horowitz, *J. Cell Biol.* **97**, 1240 (1983); I. Lang and R. Peters, in *Information and Energy Transduction in Biological Membranes*, H. J. Helmreich, Ed. (Liss, New York, 1984), pp. 377–386].
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Phosphatidylinositol-Glycan Anchors of Membrane Proteins: Potential Precursors of Insulin Mediators

GUILLERMO ROMERO, LOUIS LUTTRELL, ALAN ROGOL, KONRAD ZELLER, ERIK HEWLETT, JOSEPH LARNER

BC_3H1 myocytes release membrane-bound alkaline phosphatase to the incubation medium upon stimulation with insulin, following a time course that is consistent with the generation of dimyristoylglycerol and the appearance of a putative insulin mediator in the extracellular medium. The use of specific blocking agents shows, however, that alkaline phosphatase release and dimyristoylglycerol production are independent processes and that the blockade of either event inhibits the production of insulin mediator. These experiments suggest a new model of insulin action.

EARLY WORK ON INSULIN MEDIATORS suggested that they contained amino acids and carbohydrates and thus were termed glycopeptides (*1*). It was then suggested that they might arise from glycoproteins by proteolysis (*1*). More recent work has suggested that some of the putative mediators of insulin action may originate from membrane lipids containing inositol, sugars, phosphate, and saturated fatty acids (*2–4*). These precursors have been found in various cell membranes (*2–4*) and bear a remarkable resemblance to the glycophospholipid anchor of a number of exofacial cell membrane proteins, among which alkaline phosphatase (AP) was the first to be described (*5–9*). As suggested by this similarity, we propose that the glycophospholipid anchor of membrane proteins may serve as a precursor of insulin mediators. According to this hypothesis, (i) insulin should stimulate the release of glycophospholipid-anchored proteins from cell membranes; (ii) this release must be kinetically coupled with the production of insulin mediator; (iii) mediator production should occur, at least in part, on the outside of the cell; and (iv) at least two independent hydrolytic activities must be present in order to generate insulin mediator. We now report the results of our observations on the effects of insulin and insulin action blockers on the release of AP and insulin mediator and on the generation of [^{3}H]myristate-labeled diacylglycerol.

Treatment of BC_3H1 myocytes with insulin rapidly increases the activity of AP in the culture medium (Fig. 1a), reaching a maximum within the first 2 minutes of incubation. This process is accompanied by a significant loss (up to 80%) of the cell-associated AP activity (*10*). The AP released to the medium disappears rapidly and decreases to control levels after 5 minutes. This suggests that the enzyme is (i) rapidly inactivated after solubilization, (ii) taken up by the myocytes after its release, or (iii) rapidly bound to some specific sites in the cell membrane such as those described by Ishihara *et al.* with heparan sulfate proteoglycan (*11*). Inactivation of the enzyme was suggested as the most likely explanation on the basis of similar observations made with purified liver membranes (*10*). This alternative is also consistent with a significant decrease in the activity of AP associated with brain microvessels within 5 minutes of exposure to insulin (*12*).

The activity of insulin mediator in the incubation medium was examined with a pyruvate dehydrogenase (PDH) activation assay (*13*). These experiments were based on the following rationale. If insulin mediator originates from the glycophospholipid anchor region of membrane proteins, then, because these proteins face the extracellular medium, insulin mediator production should occur primarily at the outer layer of the cell membrane and mediator should be released rapidly to the extracellular medium. To investigate this possibility, we collected culture medium at specific times after stimulation of cells with insulin and purified the mediator by chromatography on AG-1 ion-exchange columns eluted with dilute HCl (*14*). Fractions were collected, concentrated

G. Romero, L. Luttrell, K. Zeller, J. Larner, Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, VA 22908.
A. Rogol, Departments of Pediatrics and Pharmacology, University of Virginia School of Medicine, Charlottesville, VA 22908.
E. Hewlett, Departments of Internal Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville, VA 22908.

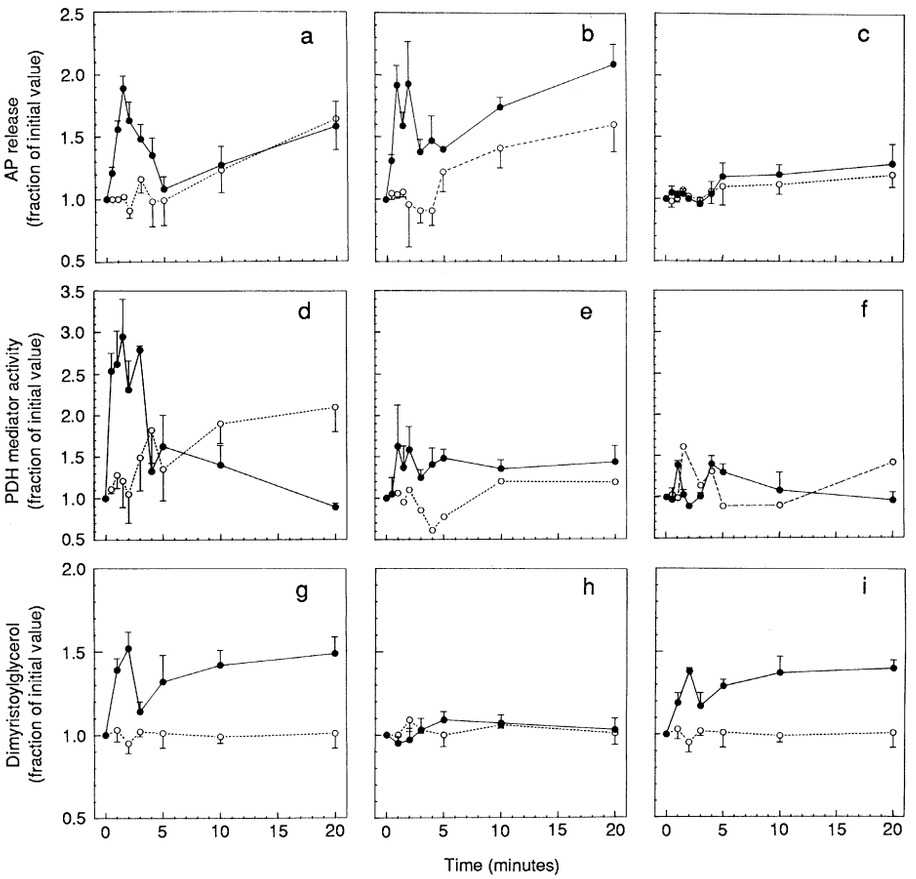

Fig. 1. The time course of the effects of insulin on the release of (AP) (a to c), release of insulin mediator to the incubation medium (d to f), and generation of dimyristoylglycerol (g to i). BC_3H1 myocytes were cultured as described (*30*), serum starved for 20 hours, and stimulated with 100 n*M* insulin in serum-free medium containing 0.1% bovine serum albumin. All experiments were carried out at 37°C at least in triplicate. Results are expressed as fraction of the initial value of the parameter measured in order to facilitate comparisons among the various experiments. The inhibitors used (PAB and PT) had no significant effect on basal activities. In all the graphs (●) indicates the presence of insulin, and (○) the absence of insulin. (**a**) The release of AP to the incubation medium. At the times indicated, portions of the culture medium were withdrawn and frozen in liquid nitrogen. The activity of AP in the medium was estimated from the rate of hydrolysis of *p*-nitrophenylphosphate (2 mg/ml) at 37°C in 0.1*M* tris (*p*H 9.2) containing 2 m*M* $MgCl_2$. The reaction was monitored spectrophotometrically at 410 nm, and the rate of hydrolysis of the substrate was determined from the slope of the time course of the reaction. (**b**) The release of AP from PT-treated BC_3H1 cells. PT was purified as described (*31*). Cells were exposed to PT (100 ng/ml) for 20 hours before insulin stimulation. (**c**) The effect of PAB on the release of AP. The inhibitor (0.8 m*M*) was added to the culture medium 2 to 4 minutes before exposure to insulin. (**d**) The activity of insulin mediator released to the extracellular medium. Mediator activity was determined in 0.2-ml portions of the culture medium collected at the indicated times with a PDH stimulation assay as described elsewhere (*13*). The samples were chromatographed in 2-ml AG-1 columns eluted successively with 10 ml of distilled water and three solutions of aqueous HCl adjusted at *p*H 3, 2, and 1.3, respectively (*14*). The eluates were concentrated by lyophilization, suspended in water, and lyophilized. Mediator activity was found in the *p*H 2 and *p*H 3 fractions. (**e**) The effect of PT treatment on the release of mediator to the culture medium. (**f**) The effect of PAB (0.8 m*M*) treatment (2 to 4 minutes) on the release of insulin mediator. (**g**) The production of dimyristoylglycerol by myocytes. Dimyristoylglycerol production was determined as described (*15*). (**h**) The effect of PT on the production of dimyristoylglycerol. (**i**) The effect of PAB in the generation of dimyristoylglycerol.

by lyophilization, and assayed for their activity to stimulate pyruvate dehydrogenase in purified mitochondria (*13*). We found that insulin treatment induced the release of a PDH activator to the medium. This mediator has not been further purified, and therefore its chemical structure is still to be determined. However, its chromatographic behavior is indistinguishable from that of an essentially homogeneous insulin PDH mediator isolated from rat liver (*14*). The activity of both mediators is destroyed by nitrous acid deamination (*10*), indicating the presence of an amino sugar–carbohydrate glycosidic linkage. This suggests that both mediators are similar and structurally related to the inositol-glycans described by Saltiel *et al.* (*2, 3, 15*) and Mato *et al.* (*4*). The concentration of PDH mediator in the medium increased immediately after the addition of insulin (Fig. 1d). The time course of this process suggests that the observed mediator release is functionally significant and not a simple consequence of the diffusion of mediator accumulated inside the cell. Furthermore, after reaching a maximum, the concentration of mediator decreased rapidly, suggesting the presence of a mediator transport mechanism. In contrast, a mediator-like activity that accumulated with time appeared slowly in the culture medium of untreated cells. These observations suggest that both mediator production and transport are modulated by insulin.

We have shown that insulin stimulates the release of both AP and PDH mediator into the extracellular medium with a kinetic profile which suggests that the two events are directly related. Our initial hypothesis and various reports on the chemical nature of insulin mediators (*2–4, 14, 15*) led us to examine the possible correlation between the release of AP and insulin mediator and the activity of phospholipase C. For this purpose, we studied the effects of insulin on the production of dimyristoylglycerol on the assumption that it reflects, as suggested by Saltiel *et al.* (*15*), the activation of phospholipase C by insulin in BC_3H1 myocytes.

Insulin stimulates rapid generation of dimyristoylglycerol (Fig. 1g) (*15, 16*). The time course of the process is consistent with the kinetics of release of AP and PDH mediator, an indication that the three processes may be related. This was further investigated by treatment of the BC_3H1 myocytes with *Bordetella pertussis* toxin (PT). We showed that treatment of BC_3H1 cells with PT (100 ng/ml) blocks the early insulin-stimulated generation of dimyristoylglycerol as well as other insulin-mediated cellular effects (*16*). These results were confirmed by our present data (Fig. 1h). In contrast, PT had no significant effect on the early release of AP (Fig. 1b). Thus, AP release and dimyristoylglycerol production are simultaneous but independent events, probably catalyzed by different hydrolytic enzymes.

The involvement of proteases in the early stages of insulin action has been suggested by several investigators (*1, 17–21*). To test the possible participation of a proteolytic reaction on the release of AP, we studied the effects of the protease inhibitor *p*-aminobenzamidine (PAB). This agent is a potent blocker of insulin action in rat adipocytes (*17*), and preliminary experiments showed that insulin-stimulated PDH activation is inhibited by PAB in BC_3H1 cells (*10*). PAB (0.8 m*M*) blocks the insulin-stimulated re-

lease of AP (Fig. 1c). Under identical conditions, PAB had no significant effect on the production of dimyristoylglycerol (Fig. 1i). These experiments show that the effect of insulin on the release of AP is apparently independent of a parallel stimulation of phospholipase C. These two activities are, nevertheless, directly associated with the production of mediator in the extracellular medium (Fig. 1, e and f); both PT and PAB inhibited the release of insulin mediator.

These results demonstrate (i) that insulin stimulates the release of AP, (ii) that the time course of this release parallels the kinetics of dimyristoylglycerol and insulin mediator generation, (iii) that the effects of insulin on the production of dimyristoylglycerol and the release of AP can be blocked independently by inhibitors of insulin action under conditions in which these inhibit well-characterized insulin-mediated effects, and (iv) that the inhibition of either event blocks the release of an insulin mediator to the extracellular medium. The simplest mechanism consistent with these findings is that insulin stimulates two independent hydrolytic enzymes. One is a protease-like enzyme that is inhibited by PAB and cleaves the glycophospholipid anchor of membrane proteins in a region distal to inositol. This cleavage is primarily responsible for the release of AP and probably of other membrane proteins. The second hydrolytic enzyme is a phospholipase sensitive to PT. This enzyme is responsible for the increased dimyristoylglycerol production observed during the early phases of insulin action. As a consequence of the action of these hydrolases, a low molecular weight substance with insulin-mediator properties is released to the extracellular medium and then transported into the cell by a mechanism whose nature remains to be clarified. This model is consistent with our data and with a number of other observations. Insulin-mediated activation of specific phospholipases C has been reported (*15, 16*), and a phospholipase with the appropriate specificity has recently been purified (*22*). The involvement of proteases in the action of insulin has also been reported by direct demonstration of insulin-stimulated proteolytic activity (*18*) and by the blockade of insulin-mediated effects, including mediator production, by certain protease inhibitors (*17, 19–21*). Finally, the cellular transport of mediator has been shown on intact cells by the demonstration of insulin-mimetic effects of semipurified mediator preparations added to the extracellular medium (*23–26*), effects that can be blocked by the addition of inositol phosphate to the medium (*27*). This model represents a novel mechanism of hormone action.

The observed apparent dissociation between phospholipase activity and AP release requires additional clarification, since AP is bound to the membrane by a phosphatidylinositol-glycan anchor and is solubilized by bacterial phospholipases C (*5*). PAB treatment blocked the release of AP by insulin under conditions where no effects of the inhibitor on the generation of myristate-labeled diacylglycerol were detected. Since, under these conditions, phospholipase C appears to be fully active, at least some phospholipase C–mediated release of AP should have been observed. The simplest explanation of this apparent inconsistency is that intact AP is a poor substrate for the specific phospholipase C involved in insulin action, so that the hydrolysis of the AP anchor requires the release or degradation of the protein before phospholipase action. Consistent with this idea are the substantial differences found in the sensitivity of glycophospholipid-anchored membrane proteins to solubilization by bacterial phospholipases C (*28*). Nevertheless, two alternative explanations may also be considered. First, AP release might result from phospholipase activation whereas, as proposed by Farese and co-workers (*29*), most of the dimyristoylglycerol generated during the burst may be a consequence of de novo lipid synthesis. Second, AP release and dimyristoylglycerol production might be related to the action of two different phospholipases, so that the two events are coincidental but unrelated phenomena. In these two cases, however, precursors that allow rapid release of mediator to the outside of the cell and a PAB-sensitive event involved in the generation of mediator must be identified. The most likely candidates to fill these blanks are, as proposed by the model, glycophospholipid-anchored proteins and proteolysis, respectively.

It may be argued that the proposed model is not economical since it would involve the degradation of macromolecules in the generation of the low molecular weight second messengers of insulin action. However, it is generally admitted that insulin generates a cascade of events involving phosphorylation and dephosphorylation of polypeptides and the generation of second messengers by mechanisms that are related to insulin receptor phosphorylation (*13*). Mediator generation is necessarily one of the initial processes of this cascade. Therefore, a small number of mediator molecules per cell may be required to initiate insulin action and, in consequence, only a small number of membrane proteins may be degraded in the generation of mediator. The proposed mechanism, therefore, does not necessarily involve any additional waste. Furthermore, the model indicates a mechanism by which insulin action may be amplified at the cellular level. By providing a mechanism to transmit signals to the extracellular medium, the model suggests a possible paracrine action of insulin by means of which cells lacking an insulin receptor, but containing a system for the transport or diffusion of the mediator to the cytoplasm, may be indirectly stimulated by insulin.

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