sampled and as the system responds to changing enthalpy conditions over long periods of time. Because of road destruction, Termales de Ruiz was the only well-characterized hot spring accessible in November. The 1969 to 1985 differences are not easily explained by sampling differences or by analytical error.
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Insulin-Stimulated Hydrolysis of a Novel Glycolipid Generates Modulators of cAMP Phosphodiesterase

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Insulin action may involve the intracellular generation of low molecular weight substances that modulate certain key enzymes. The production of two substances that regulate the activity of adenosine 3′,5′-monophosphate phosphodiesterase was evaluated in cultured myocytes by incorporation of radiolabeled precursors. Insulin caused the rapid hydrolysis of a chemically undefined membrane glycolipid, resulting in the production of two related complex carbohydrates as well as diacylglycerol. Both the glycolipid precursor and the aqueous products were monitored by labeling with radioactive inositol and glucosamine. Depletion of the labeled precursor and the appearance of labeled water-soluble products and diacylglycerol occurred within 30 seconds after hormone treatment and was followed by rapid resynthesis of the precursor. The aqueous products that were radioactively labeled appeared chromatographically and electrophoretically identical to phosphodiesterase modulating activities produced by insulin from the same cells. The purified radiolabeled and bioactive substances had similar chemical properties. Hydrolysis of the glycolipid precursor and subsequent generation of products could be reproduced by incubation of extracted lipids with a phosphatidylinositol-specific phospholipase C. These studies suggest that insulin stimulates an endogenous, selective phospholipase C activity that hydrolyzes a novel glycolipid, resulting in the generation of a complex carbohydrate-phosphate substance containing inositol and glucosamine that may mediate some of the actions of the hormone.

THE MOLECULAR MECHANISMS OF insulin action remain largely unexplained. Insulin binds to a heterodimeric cell surface receptor that contains a hormonally responsive tyrosine kinase activity in its β subunit (*1*). The ensuing changes in cellular metabolism are diverse, occurring within seconds or hours of the hormone-receptor interaction. The regulation by insulin of enzymes controlling intermediary metabolism is observed within minutes and is often the result of changes in the state of phosphorylation of the enzyme. However, the precise biochemical link between the activated receptor and enzyme regulation remains unknown. Several known substances have been proposed to act as second messengers for insulin, yet each has proved inadequate to account for the action of the hormone (*2*). Recently, investigators have found (*3*) that some of the metabolic effects of insulin may result from the generation from the plasma membrane of an undefined substance or group of substances that regulate certain insulin-sensitive enzymes, perhaps through control of protein phosphorylation (*4*). These "mediator" activities were shown to be associated with a substance, or substances, that were water-soluble, nonnucleotide, and had an apparent molecular weight of 1000 to 2000 (*3, 4*). Although there were suggestions of a peptidic structure (*3, 4*), definitive information concerning the chemical identities of these substances has not been forthcoming.

We recently reported (*5*) the purification and partial characterization of two structurally related complex carbohydrate-phosphate substances produced from liver membranes that regulated the activity of the high-affinity adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase (PDE) in adipocytes. Both substances were apparently generated by exposure of membranes to insulin, and appeared to result from the phosphodiesterase-like cleavage of a novel inositol-containing glycolipid. Studies on enzymatic production of these activities with a phosphatidylinositol-specific phospholysine C (PI-PLC), and evaluation of chemical properties, suggested that these enzyme modulating activities were derived from substances that contained inositol and glycosidically linked glucosamine.

In the present studies the actions of insulin on the production of these carbohydrate substances were evaluated in the cultured murine myocyte line BC_3H1 (*6*). These cells become responsive to insulin when they differentiate after attaining confluence. We observed the insulin-sensitive incorporation of radioactive inositol and glucosamine into fractions separated by high-performance liquid chromatography (HPLC) that contain PDE-modulating activity. The production of these radioactive products mirrors that of the PDE-modulating activities, and appears to be due to the hydrolysis of a novel glycolipid precursor.

The purification of two liver-derived substances capable of modifying the activity of the high-affinity cAMP phosphodiesterase from adipocyte particulate fraction was recently reported (*5*). This procedure was modified for purification of similar substances from BC_3H1 cells incubated with [^{3}H]inositol or [^{3}H]glucosamine. After in-

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sulin treatment and extraction, the aqueous phase was sequentially chromatographed on DEAE-cellulose, C-18 reversed-phase resin, and Dowex 50WX-4 cation exchange. The eluate was then subjected to chromatography on a SAX HPLC column (Fig. 1A). Insulin treatment increased the incorporation of [^{3}H]inositol into two peaks that eluted at 15 (I) and 18 (II) minutes. Minimal incorporation into these fractions was observed in control cells. Insulin had a similar effect on incorporation of [^{3}H]glucosamine into identical fractions, although another radioactive peak was detected at 23 minutes which was unaffected by insulin. The recovery of [^{3}H]glucosamine and [^{3}H]inositol detected in these water-soluble products suggested an approximate 1:1 stoichiometry. The two peaks of insulin-stimulated radioactivity exhibited distinct retention times from those of 1,2-cyclic inositol monophosphate (10 minutes) or inositol monophosphate (24 minutes). However, peaks I and II coeluted with two peaks of PDE-modulating activity produced in these cells in response to insulin. These insulin-sensitive myocyte-derived activities were chromatographically and electrophoretically indistinguishable from those purified from insulin-treated liver plasma membranes and exhibited identical chemical properties (*5*).

Modulators of PDE can be produced from liver membranes by addition of PI-PLC from *Staphylococcus aureus* (*5*). This enzyme specifically hydrolyzes the phosphodiester linkage of phosphatidylinositol (*7*) as well as phosphatidylinositol covalently attached to proteins by way of a glycan anchor (*8*). This PI-PLC is free of protease activity, and is inactive against polyphosphoinositides and other phospholipids (*7*). BC_3H1 cells were incubated with [^{3}H]inositol or [^{3}H]glucosamine, and lipids were extracted and treated with PI-PLC. After 30 minutes of incubation, the cells were extracted and the aqueous products were purified as described above and chromatographed on a SAX HPLC column (Fig. 1B). The PI-PLC treatment resulted in the generation of two peaks containing [^{3}H]inositol that exhibited retention times identical to those produced by insulin in intact cells. The [^{3}H]glucosamine in these fractions was increased after PI-PLC treatment of extracted lipids, although an additional peak was observed at 23 minutes that was unaffected by PI-PLC.

We previously observed a chemically unidentified glycolipid in liver plasma membranes that appeared to serve as a substrate for the generation of PDE modulators by the *S. aureus* PI-PLC (*5*). The incorporation of [^{3}H]inositol and [^{3}H]glucosamine into this putative precursor was examined in BC_3H1 cells. Thin-layer chromatography of extracted phospholipids revealed a peak into which both [^{3}H]inositol and [^{3}H]glucosamine were incorporated (Fig. 2A). This glycolipid exhibited an R_F (0.22) identical to that of the putative precursor of the PDE modulator from liver (*5*). It migrated faster than phosphatidylinositol 4-phosphate but slower than phosphatidylinositol. After treatment of cells with insulin for 10 minutes, the detection of both [^{3}H]inositol and [^{3}H]glucosamine in this peak was increased approximately 40%, with no statistically significant change in labeling of other lipids. In parallel experiments, lipids were extracted from labeled cells and treated with or without PI-PLC for 30 minutes (Fig. 2B). Exposure to this enzyme caused a 60% decrease in the [^{3}H]inositol labeled spot and a 40% decrease in the [^{3}H]glucosamine labeled one. Phosphatidylinositol was 50% depleted by the *S. aureus* PI-PLC (*9*). Similar results were observed by PI-PLC treatment of intact plasma membranes prepared from these labeled myocytes.

The time course of radioisotopic incorporation into the products and putative precursor was examined after exposure of BC_3H1 cells to 10 n*M* insulin (Fig. 3A). Insulin rapidly stimulated the generation of these [^{3}H]inositol-containing products, achieving maximal production by 2 minutes. PDE-modulating activities were produced from BC_3H1 cells by insulin over the same time course and concentration range as were the purified radiolabeled products. The time course of the [^{3}H]inositol content of the precursor (as identified on thin-layer chromatography in Figs. 3 and 4) upon insulin stimulation was biphasic. Insulin caused a small (20%) but significant decrease in counts at 30 seconds. Thereafter, radioactivity in this peak increased, resulting in a 60% increase over control levels. This process

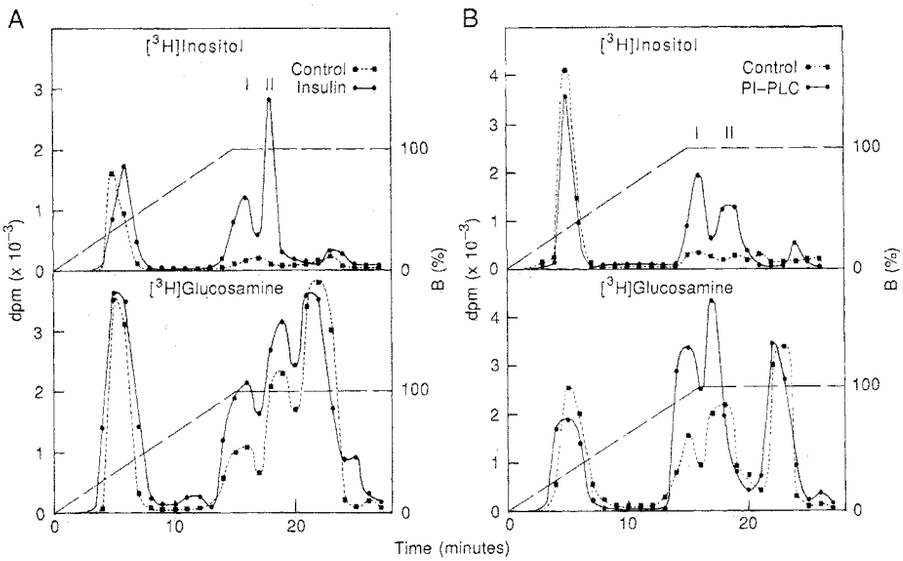

Fig. 1. Analysis of radiolabeled products by strong anion exchange (SAX) HPLC. (A) BC_3H1 cells were cultured at 37°C in Dulbecco's minimal essential medium (DMEM) in the presence of 20% NU serum (Collaborative Research) on collagen-coated 10-mm miniwells (*6*). Cells (1×10^6 per well) were incubated with 1 μCi of [2,6-^{3}H]myoinositol (top) or [1,6-^{3}H]glucosamine (bottom) (New England Nuclear) for 20 hours in the presence of 20% NU serum. Cells were treated with (●) or without (■) 10 n*M* insulin in serum-free medium. Reactions were terminated after 10 minutes by removal of medium and addition of 1 ml of a mixture of chloroform, methanol, and 1*N* HCl (200:100:1), and then 0.5 ml of 10 m*M* formic acid was added. Six wells were pooled for each extraction. Organic and aqueous phases were separated by centrifugation at 500*g* for 5 minutes. The water-soluble labeled products were purified by a modified procedure for purification of cAMP PDE modulators (*5*). The aqueous phases were chromatographed on DEAE-cellulose, eluted with 0.25*M* triethylamine (TEA)-formate, *p*H 3.75. This fraction was eluted through a C-18 reversed-phase column in the same buffer. After lyophilization, the solution was eluted through Dowex 50WX-4 in 50 m*M* TEA-formate, *p*H 3.0. After exposure to activated charcoal, samples were chromatographed on a SAX HPLC column (Whatman), eluted with a linear, 15-minute gradient of 60% methanol (A) to 0.5*M* TEA-formate, *p*H 3.75 (B), at 1 ml/min. One-milliliter fractions were counted. The two peaks of insulin-stimulated radioactivity coeluted with similarly purified, myocyte-derived PDE-modulating activities. (B) Cells were labeled as described, extracted in chloroform, methanol, and 1*N* HCl (200:100:1) and centrifuged at 1000*g* for 10 minutes. The supernatant was dried under N_2 and resuspended in 1 ml of 50 m*M* ammonium bicarbonate, *p*H 7.4. These solutions were treated with (●) or without (■) 1.0 μg/ml of PI-PLC from *S. aureus* (*7*) for 30 minutes at 37°C. Reactions were terminated by reextraction with 1 ml of chloroform, methanol, and 1*N* HCl (200:100:1) and the aqueous and organic phases were separated. The aqueous phases were then purified as above and chromatographed on a SAX HPLC column, and eluted as described. In both (A) and (B), the results are representative of a single culture, which was reproduced in several experiments.

appears similar to that observed in the hormone-stimulated phosphodiesterase-like cleavage of the phosphoinositides, in which these phospholipid substrates are rapidly resynthesized.

The phosphodiesterase-induced cleavage of a phospholipid by insulin should also be reflected by increased production of diacylglycerol, which could serve as a substrate for diglyceride kinase to produce phosphatidic acid. Myocytes were incubated with [^{3}H]myristic acid, and the incorporation of radioactivity into both diacylglycerol and phosphatidic acid was evaluated after addition of insulin (Fig. 3B). Insulin caused a 40% increase in [^{3}H]myristate diacylglycerol within 1 minute. Labeled diacylglycerol declined to near basal levels by 2 minutes and then increased, reaching a maximal 50% increase over the basal levels by 5 to 10 minutes. The production of labeled diacylglycerol was followed 5 minutes later by a twofold increase in levels of [^{3}H]myristate phosphatidic acid. [^{3}H]Myristate diacylglycerol was also rapidly produced by incubation of extracted lipids with *S. aureus* PI-PLC.

The chromatographic properties of the [^{3}H]inositol- and [^{3}H]glucosamine-labeled products from insulin-treated BC_3H1 cells were further evaluated. Pooled radioactivity

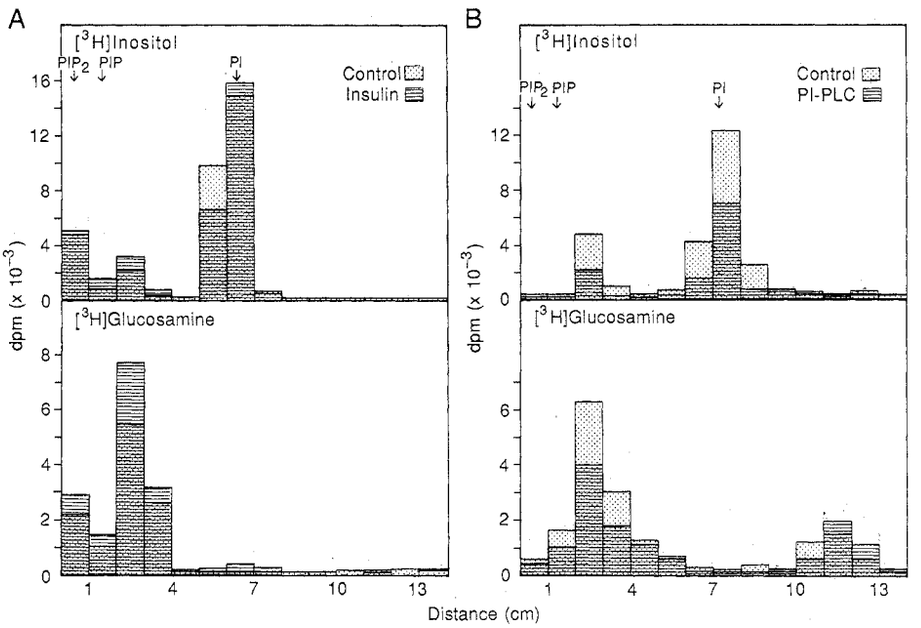

Fig. 2. Thin-layer chromatography of the radiolabeled precursor. (A) [^{3}H]Inositol- (top) and [^{3}H]glucosamine- (bottom) labeled BC_3H1 cells in 10-mm wells (six wells per incubation) were treated with or without 10 n*M* insulin for 10 minutes. After extraction and phase separation, the organic phase was dried under N_2, resuspended in chloroform, methanol, and H_2O (9:7:2), and spotted on oxalate-impregnated Silica gel G plates. These were twice developed in chloroform, acetone, methanol, glacial acetic acid, and H_2O (10:4:2:2:1). One-centimeter regions were scraped and radioactivity was determined by scintillation counting. Phosphoinositides were identified by iodine staining of standards. (B) Cells were labeled, extracted, treated with or without PI-PLC, and reextracted as described above. In both (A) and (B) the results are representative of a single culture, which was reproduced in several experiments.

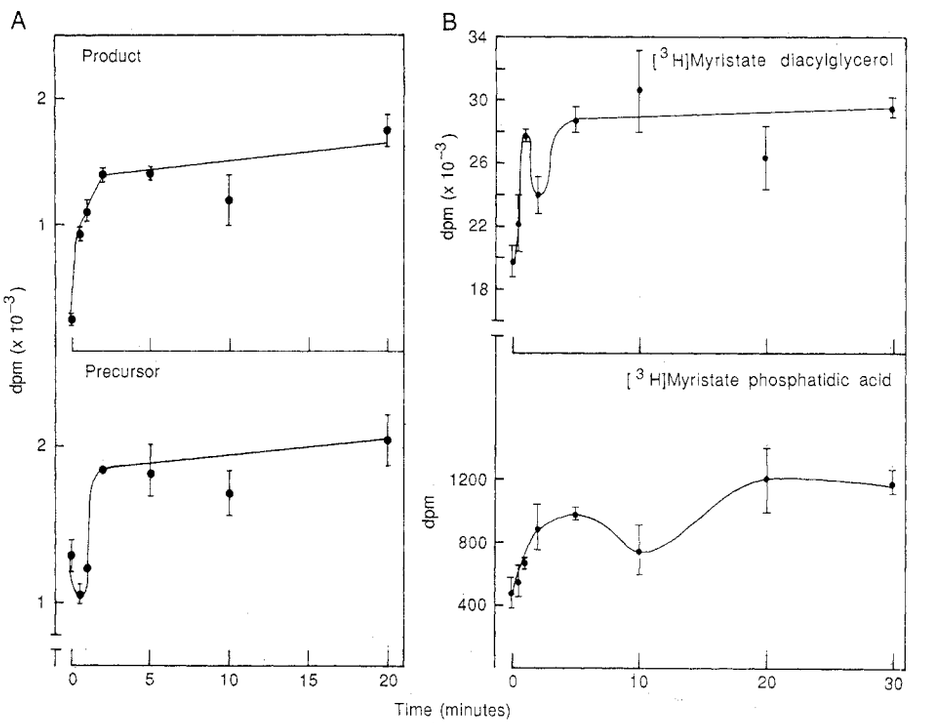

Fig. 3. Time course of precursor hydrolysis by insulin. (A) BC_3H1 cells in 10-mm multiwells were labeled with [^{3}H]inositol and treated with 10 n*M* insulin for the designated intervals, extracted, and phase-separated as detailed in Fig. 1. Aqueous phases were chromatographed up to and including the SAX HPLC step (top). Results reflect counts from a combination of peaks I and II. The ratio of counts in these peaks was unchanged over the time course. The precursor residing in the organic phases was identified by scraping and counting of the peak on TLC with an R_F = 0.22, corresponding to the species depleted by PI-PLC in Fig. 2. (B) Myocytes in 10-mm multiwells were incubated for 20 hours in serum-free DMEM with [9,10^{3}H]myristic acid (New England Nuclear) complexed 1:1 to bovine serum albumin. Insulin (10 n*M*) was added to cells in fresh medium for the designated intervals, and reactions were stopped by removal of medium and addition of 1 ml of a mixture of chloroform, methanol, and 1*N* HCl (200:100:1). 0.6 ml of H_2O was added, solutions were centrifuged at 500*g* for 5 minutes. Upper aqueous phases were discarded and the lower, organic phases were dried under N_2 and resuspended in 1 ml of diethyl ether; 1 ml of 50 m*M* formic acid was then added. The upper ether phase was aspirated and the lower phase reextracted with 1 ml of diethyl ether. The combined ether phases, containing diacylglycerol, were dried under N_2, resuspended in chloroform, and spotted on Silica gel G plates that had been activated at 60°C for 1 hour. Plates were twice developed in a mixture of petroleum ether, diethyl ether, and glacial acetic acid (70:30:2) (top). Unlabeled dimyristoyl glycerol (5 μg) was added to each sample. Spots were visualized by iodine staining. To the lower aqueous phases from the ether extraction were added 1 ml of chloroform and methanol (2:1). After centrifugation, the resulting upper aqueous phases were discarded and the lower organic phases dried under N_2, resuspended in chloroform, methanol, and H_2O (9:7:2), and spotted on "Soft Plus" (Merck) silica gel plates. These were developed in chloroform, pyridine, and 70% formic acid (50:30:7), and phosphatidic acid was identified by an iodine-stained standard (bottom). Lipids were scraped and radioactivity determined in a liquid scintillation counter. Results are expressed as the means of triplicate determinations with standard deviations.

residing in SAX peak I was chromatographed on a P-2 gel filtration column (Fig. 4A). The elution profiles of [^{3}H]inositol- and [^{3}H]glucosamine-labeled products were compared with the profiles of the similarly purified myocyte-derived PDE-modulating activity that was insulin-dependent. Each substance eluted in an identical volume, indicating an approximate molecular weight of about 1400. Both radioactive and bioactive substances in peak II exhibited an identical elution on P-2. After P-2 gel filtration, active samples were pooled and subjected to high-voltage electrophoresis on cellulose-coated thin-layer plates (Fig. 4B). The electrophoretic migration of both labeled products in peak I was identical to that of the PDE-modulator. Similar results were obtained when samples were electrophoresed at *p*H 1.9. Radioactively labeled products and PDE-modulatory activity residing in peak II migrated slightly farther than that of peak I at both *p*H values as described previously (*5*), although comigration of radioactivity and bioactivity was again observed. These results confirm previous findings (*5*) of dissimilar net negative charge for these compounds even at *p*H 1.9, implicating phosphate or sulfate as a charged species.

Differences in the two water-soluble products were further evaluated by digesting each of the [^{3}H]inositol-labeled peaks at 25°C for 16 hours with alkaline phosphatase (100 U/ml) in 50 m*M* ammonium bicarbonate, *p*H 8.0. Each sample was then injected onto a SAX HPLC column, and counts in each peak were determined. Alkaline phosphatase treatment had no effect on counts in peak I, but produced a 70% loss of counts in peak II (Table 1). These results were consistent with the possibility that peak I contains a 1,2-cyclic inositol phosphate derivative that is not susceptible to alkaline phosphatase, whereas peak II may contain an inositol monophosphate derivative that is hydrolyzed by alkaline phosphatase. Such cyclic products have been identified for the phosphodiesterase cleavage of phosphoinositides (*10*) and the phosphatidylinositol-anchored variant surface glycoprotein from *Trypanosoma brucei* (*11*). The physiological significance of compounds with cyclic inositol phosphate moieties and their relation to the noncyclic compounds remain unclear (*11*), and it is uncertain whether both products described here are generated in vivo.

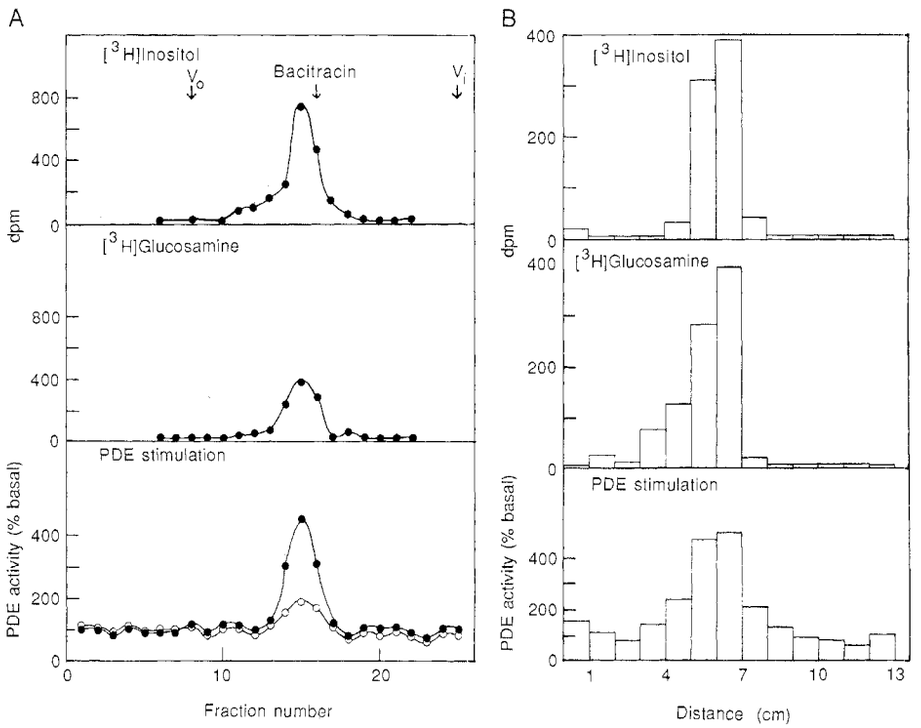

Fig. 4. Further characterization of the radioactive and bioactive products. (A) [^{3}H]Inositol- (top) and [^{3}H]glucosamine- (center) labeled substances from insulin-treated BC_3H1 cells from six 10-mm multiwells were purified up to the SAX HPLC step. Fractions eluting in peak I were chromatographed on a 45-ml P-2 gel filtration column in 50 m*M* formic acid (*5*). Unlabeled cells from six wells were treated with or without 10 n*M* insulin for 10 minutes, extracted by an identical procedure and purified up to the SAX HPLC column, and eluted as described in Fig. 1. Active fractions eluting in peaks I and II were identified by stimulation of the low K_m cAMP phosphodiesterase (PDE) activity. This was assayed in adipocyte particulate fractions (*21*) as detailed (*5, 22*). Control (○) and insulin-stimulated (●) PDE-modulating activities in peak I were chromatographed on the P-2 column (bottom). Fractions were collected and counted (top and center) or assayed (bottom) for PDE-modulating activity. Radioactivity and bioactivity residing in peak II from SAX exhibited an identical elution volume on this column. (B) After gel filtration, purified radioactive and bioactive fractions from insulin-treated cells residing in SAX peak I were subjected to high-voltage electrophoresis on cellulose-coated thin-layer plates at 500 V for 1 hour (*5*). The *p*H of the running buffer was 3.5 [pyridine, glacial acetic acid, and H_2O (1:10:189)]. One-centimeter regions were scraped and eluted with 50% methanol in 10 m*M* formic acid. [^{3}H]Inositol and [^{3}H]glucosamine labeled samples were counted. Bioactive samples were assayed for PDE-modulating activity (bottom). Radioactive and bioactive samples exhibited identical migrations when electrophoresed at *p*H 1.9 [88% formic acid, glacial acetic acid, and H_2O (50:56:1794)]. Radioactivity and bioactivity residing in peak II similarly migrated slightly farther from the origin at both *p*H 1.9 and 3.5, as described previously (*5*). Results are representative of a single culture and were reproduced in several experiments.

It is known that treatment with nitrous acid at *p*H 3.75 deaminates glycosidically linked glucosamine, converting the residue to a 2,5-anhydromannose derivative involving cleavage of the glycosidic linkage (*12*). Treatment of the purified [^{3}H]inositol-labeled products in both peaks with nitrous acid caused an 80% loss of counts detected after HPLC (Table 1). These results were verified by measurement of radioactivity on high-voltage electrophoresis. Eighty-five percent of [^{3}H]inositol migrated as inositol monophosphate after nitrous acid deamination.

Upon differentiation, the cultured myocyte line BC_3H1 develops high-affinity insulin receptors and several biological responses to the hormone, including glucose transport, amino acid uptake, insulin receptor down-regulation, and increased labeling of certain phospholipids (*6, 13*). We have described a novel pathway in these cells that may mediate some of the actions of insulin, the apparent hydrolysis of a phosphoinositide-glycan precursor that results in the production of two water-soluble carbohydrate substances as well as diacylglycerol. The generation of these products was reproduced by treatment of extracted phospholipids with *S. aureus* PI-PLC, which specifically hydrolyzes phosphatidylinositol (*7*) and releases proteins covalently anchored to phosphatidylinositol (*8*). These findings suggest that insulin may activate an endogenous PLC activity that hydrolyzes a novel inositol- and glucosamine-containing glycolipid. Since insulin does not stimulate hydrolysis of polyphosphoinositides (*14*), the insulin-sensitive PLC may be similar to a PLC activity identified by *Trypanosoma brucei* that selectively attacks the glucosamine-inositol phosphodiester anchor of the membrane-bound variant surface glycoprotein, but

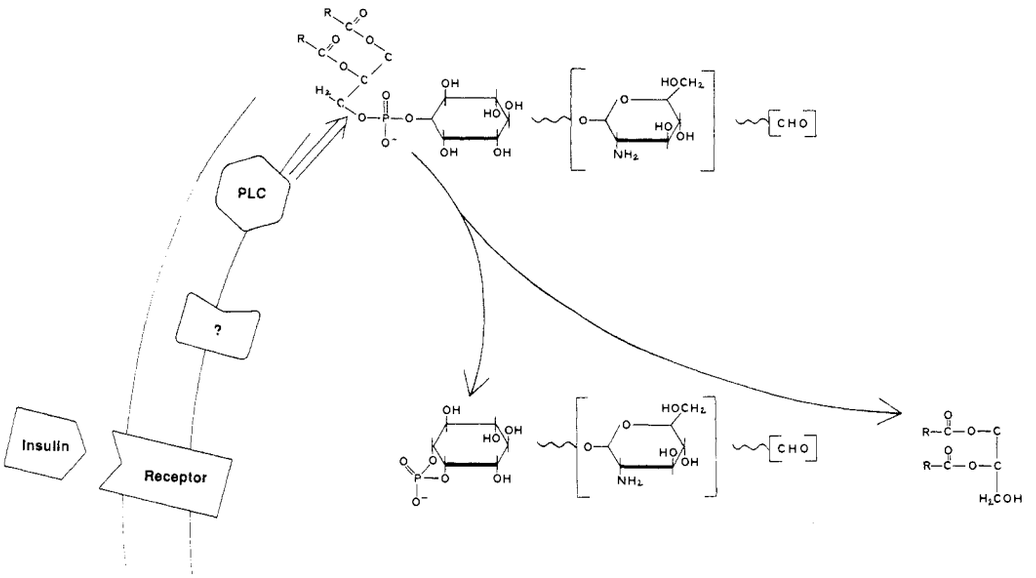

Fig. 5. Model for insulin stimulation of a phosphoinositide glycan-specific phospholipase C. Some of the actions of insulin may be mediated by stimulation of the phosphodiesterase-catalyzed hydrolysis of a novel phosphatidylinositol-containing glycolipid. The binding of insulin to its receptor is linked, perhaps through a coupling protein, to the activation of a phospholipase C that selectively hydrolyzes a phosphatidylinositol-glycan containing glucosamine and other carbohydrates. This hydrolysis results in the generation of two potential signals: (i) an inositol-phosphate glycan that regulates cAMP phosphodiesterase and perhaps other insulin-sensitive enzymes, and (ii) diacylglycerol that may selectively regulate the activity of protein kinase C or an analogous protein kinase.

does not hydrolyze other phosphoinositides (*15*). The involvement of such a selective PLC in insulin action is supported by observations that the purified trypanosome PLC is as effective as the *S. aureus* enzyme in hydrolysis of the myocyte glycolipid precursor (*16*).

Characterization of the water-soluble products from this reaction with respect to net charge (ion exchange), size (gel permeation), mass to charge ratio (high-voltage electrophoresis), and hydrophobicity (reversed phase) indicates that labeled inositol and glucosamine are coincorporated in an insulin-dependent manner. Although it is possible that these labeled precursors were converted to other sugars during the first incubation period, the incorporation of intact myo-inositol was supported by the selective hydrolysis of the putative precursor and generation of product by a PI-PLC. Glucosamine incorporation was validated by loss of radioactivity in purified fractions after nitrous acid deamination under conditions specific for cleavage of glycosidically linked glucosamine. The insulin-sensitive ^{3}H-labeled products cochromatographed in all systems tested with insulin-stimulated PDE modulators derived both from BC_3H1 myocytes and liver plasma membranes (*5*). In addition, the generation of both bioactive and labeled products with *S. aureus* PI-PLC, as well as identical chemical properties, indicates that the activities of the insulin-generated PDE modulators can be ascribed to the radiolabeled complex carbohydrates described here.

Table 1. Modification of [^{3}H]inositol-labeled products. After gel filtration, purified [^{3}H]inositol-labeled products (obtained from two 100-mm petri dishes of insulin-treated cells, 5×10^7 cells per disk) in peaks I and II were treated with (i) alkaline phosphatase (100 U/ml) in 50 m*M* ammonium bicarbonate, *p*H 8.0 for 16 hours at 30°C, or (ii) 0.2*M* sodium nitrite in 50 m*M* ammonium acetate, *p*H 3.75, for 3 hours at 25°C. After incubation, samples were injected into a SAX HPLC column and eluted as detailed in Fig. 1, and the collected fractions were counted. In control samples that were incubated in buffer alone, all counts were recovered with the appropriate retention time. Results are representative of a single incubation and were reproduced several times.

Treatment	Radioactivity (dpm)	
	Peak I	Peak II
None	1820	1100
Alkaline phosphatase (100 U/ml)	1963	341
Sodium nitrite (0.2*M*, *p*H 3.75)	220	195

These data support a model (Fig. 5) for insulin action in which hormone-receptor binding is coupled to the phosphodiesterase-like cleavage of a novel glycan phosphoinositide, resulting in the generation of a cyclic inositol phosphate-glycan that modulates PDE activity, and diacylglycerol, which is an endogenous activator of protein kinase C (*17*). Although, in previous reports, diacylglycerol production by insulin in BC_3H1 cells was attributed to stimulation of de novo synthesis (*18*), we have observed the transient generation of diacylglycerol in response to insulin followed by the apparent rapid phosphorylation to phosphatidic acid. Diacylglycerol and the [^{3}H]inositol-labeled products appeared to arise from the same precursor (*19*), and their production was kinetically indistinguishable. Moreover, the insulin-sensitive species of diacylglycerol produced rapidly in BC_3H1 cells was predominantly labeled with myristic acid. This species of diacylglycerol is not generated by agonists that stimulate hydrolysis of the phosphoinositides in these cells (*19*). It is possible that this pathway of diacylglycerol production may provide a selective mechanism for insulin's regulation of protein kinase C activity, and perhaps explain why phorbol esters reproduce some but not all of the effects of insulin (*18, 20*). The further characterization of the molecular components involved in this process, as well as the chemical identities of the aqueous products, may elucidate many of the molecular mechanisms of insulin action.

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Measurement of Single Channel Currents from Cardiac Gap Junctions

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Cardiac gap junctions consist of arrays of integral membrane proteins joined across the intercellular cleft at points of cell-to-cell contact. These junctional proteins are thought to form pores through which ions can diffuse from cytosol to cytosol. By monitoring whole-cell currents in pairs of embryonic heart cells with two independent patch-clamp circuits, the properties of single gap junction channels have been investigated. These channels had a conductance of about 165 picosiemens and underwent spontaneous openings and closings that were independent of voltage. Channel activity and macroscopic junctional conductance were both decreased by the uncoupling agent 1-octanol.

CELL-TO-CELL COMMUNICATION IS mediated by gap junctions in many tissues (*1*). Studies of the passive electrical properties of atrial and ventricular trabeculae (*2*), nodal tissue (*3*), and Purkinje fibers (*4*) have shown that all parts of the heart form an electrical syncytium of coupled cells. In all of these studies, indirect evidence has been used to argue that gap junctions mediate the electrotonic coupling. Evidence has included measurements of the diffusion of intracellular ions and tracer molecules from cell to cell (*5*), and ultrastructural analysis showing that the specialized membrane structures between cardiac fibers resemble the permeable junctions of other tissues (*6*). The macroscopic conductance of gap junctions has been determined in pairs of adult heart cells (*7–9*). However, it has not heretofore been possible to record the activity of putative gap junction channels between myocardial cells, or to measure their individual conductance or kinetic properties.

Spray *et al.* (*10*) introduced the use of two separate voltage-clamp circuits to measure directly the junctional conductance between pairs of amphibian blastomeres. A similar approach has been applied to pairs of adult mammalian heart cells (*7–9*) isolated by standard methods (*11*). However, in these experiments, the activity of single gap junction channels could not be measured because the signal-to-noise ratio in such cell pairs is quite low. If gap junction channels have an estimated resistance of 10 Gohm (*12*), only 4 pA of current would flow through an open channel between a pair of cells with a 40-mV transjunctional potential (V_j). Metzger and Weingart (*8*) found that a pair of adult ventricular cells typically had an input resistance (R_m) of about 35 Mohm. About 1200 pA of holding current would be needed to produce the required 40-mV difference in the membrane potential (V_m) between the members of such a cell pair. That is, the opening of a single junctional channel would result in an increase over the background current of about 0.3%, an amount that would be lost in the current noise. In contrast, small spheroidal cells such as those from embryonic heart and other tissues have input resistances in the gigaohm range (*13, 14*). With patch-clamp electrodes in the whole-cell recording configuration (*15*) holding current are only a few picoamperes. Against this background, a 4-pA junctional channel current is easily recognized. Using pairs of rat lacrimal gland cells, Neyton and Trautmann (*14*) measured the conductance of junctional channels but the single channel events were not apparent. With heart cell pairs, we have recorded quantal changes in junctional conductance on the order of 165 pS that correspond to the opening and closing of single gap junction channels.

Dissociation of the ventricles of 7-day chick embryos by techniques previously described (*16*) yielded a suspension of single cells containing a small percentage of cell pairs and clusters. Pairs comprised two sin-

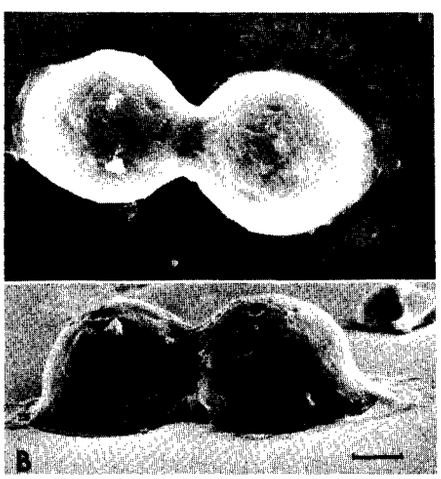

Fig. 1. Scanning electron micrographs of a pair of 7-day chick ventricle cells typical of those used in our study. (A) Top view. (B) Cells tilted 70° from vertical. Scale = 2 μm. After dissociation in a solution containing crystalline trypsin (50 μg/ml), deoxyribonuclease (5.5 μg/ml), and bovine serum albumin (fraction V, fatty acid poor, 1 mg/ml), cells were washed in medium and incubated for 24 hours in vitro in a humidified gassed atmosphere of 85% N_2, 10% O_2, and 5% CO_2. The culture medium contained (by volume) 25% M199, 2% heat-inactivated horse serum, 4% fetal bovine serum, 67.5% potassium-free Ham's F-12, 1% L-glutamine, 0.5% penicillin-G, and 4.3 m*M* KCl. Cells adhering to a chip of polylysine-coated plastic (Thermonox) were washed five times in 37°C balanced salt solution (BSS) containing 142 m*M* NaCl, 2.5 m*M* KCl, 0.8 m*M* $MgSO_4$, 0.9 m*M* NaH_2PO_4, 1.8 m*M* $CaCl_2$, 5.5 m*M* dextrose, and 10 m*M* Hepes (*p*H 7.4). They were fixed in 1.0% glutaraldehyde in BSS, stained in 1% osmium tetroxide, dehydrated from ethanol to Freon-113 by the exchange method (*25*), and critical-point dried. This treatment produced a shrinkage of about 30%. After a 5-nm layer of gold palladium was deposited on the cell surface, the cells were photographed in an ISI Model DS-130 scanning electron microscope fitted with a LAB-6 lanthanum hexaboride electron emitter. (Courtesy of R. Apkarian, SEM Laboratory, Yerkes Regional Primate Research Center, Emory University.)

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