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Reciprocal Control of Pyruvate Dehydrogenase Kinase and Phosphatase by Inositol Phosphoglycans

*DYNAMIC STATE SET BY "PUSH-PULL" SYSTEM**[S]

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Reversible phosphorylation of proteins regulates numerous aspects of cell function, and abnormal phosphorylation is causal in many diseases. Pyruvate dehydrogenase complex (PDC) is central to the regulation of glucose homeostasis. PDC exists in a dynamic equilibrium between de-phospho-(active) and phosphorylated (inactive) forms controlled by pyruvate dehydrogenase phosphatases (PDP1,2) and pyruvate dehydrogenase kinases (PDK1–4). In contrast to the reciprocal regulation of the phospho-/de-phospho cycle of PDC and at the level of expression of the isoforms of PDK and PDP regulated by hormones and diet, there is scant evidence for regulatory factors acting *in vivo* as reciprocal "on-off" switches. Here we show that the putative insulin mediator inositol phosphoglycan P-type (IPG-P) has a sigmoidal inhibitory action on PDK in addition to its known linear stimulation of PDP. Thus, at critical levels of IPG-P, this sigmoidal/linear model markedly enhances the switchover from the inactive to the active form of PDC, a "push-pull" system that, combined with the developmental and hormonal control of IPG-P, indicates their powerful regulatory function. The release of IPGs from cell membranes by insulin is significant in relation to diabetes. The chelation of IPGs with Mn^{2+} and Zn^{2+} suggests a role as "catalytic chelators" coordinating the traffic of metal ions in cells. Synthetic inositol hexosamine analogues are shown here to have a similar linear/sigmoidal reciprocal action on PDC exerting push-pull effects, suggesting their potential for treatment of metabolic disorders, including diabetes.

Pyruvate dehydrogenase complex (PDC),[2] an enzyme at the interface between glycolysis and the citric acid cycle, is influenced by dietary and hormonal control and by phosphorylation/dephosphorylation reactions, the former regulated by pyruvate dehydrogenase kinases (PDK1–4) and the latter by dedicated mitochondrial pyruvate dehydrogenase phosphatases (PDP1,2) (1–4). Phosphorylation forms the basis of the dynamic state of cell cycling networks, thus the balance between the active (de-phospho-) and the inactive (phospho-) forms of PDC is dependent upon the regulation of PDK and PDP (2, 5, 6). Cycling between two phosphorylated states is, classically, one mode of control, permitting rapid alterations in catalytic activity, *e.g.* in response to insulin, adrenaline, shifts in Ca^{2+} distribution, and effector molecules. In addition, adaptive changes due to altered hormonal or dietary states, such as diabetes, starvation, or high fat/high carbohydrate diets, and related changes in the expression of isoforms of PDK and PDP in a tissue-specific manner regulate the phosphorylation state of the PDC (2, 7–12). The profile of the regulation of PDK1–4 to activation by NADH and to NADH plus acetyl CoA and ATP, together with differences in the apparent K_i values for ADP, confers upon tissues individual patterns of response to alterations in metabolite profile linked to hormonal and dietary changes (2, 3, 9–12).

Inositol phosphoglycans (IPGs) are broadly divided into two families by separation on Amberlite columns, the IPG-P (eluting at pH 2.0) activates PDP, and the IPG-A (eluting at pH 1.3) acts upon cAMP-linked enzymes and activates acetyl-CoA carboxylase; their wide range of activities has been extensively reviewed (13–19). There is a fall in the tissue content, serum levels, and excretion of IPG-P and shifts in the IPG-P/IPG-A quotient in human diabetes type 2 and in experimental diabetes (20–23). Of particular importance in the present context is the interaction of the two classes of IPGs in the regulation of PDP, IPG-A counteracting the stimulatory effect of IPG-P (23).

The main objective of this study was to establish whether IPG-P extracted from liver and, critically, whether synthetic inositol hexosamine derivatives had reciprocal effects on PDP and PDK and thus played a dual role by activating the dephosphorylation and inhibiting the rephosphorylation of PDC, in effect a "push-pull" mechanism facilitating rapid alterations in PDC activity. The effects of IPG-P from liver, and the action of Mn^{2+} and Zn^{2+}, trace metals associated with IPGs (24, 25), were examined for their effects on PDK. The results indicated

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[2] The abbreviations used are: PDC, pyruvate dehydrogenase complex; DCA, dichloroacetate; IPG-A, inositol phosphoglycan A type; IPG-P, inositol phosphoglycan P type; PDK 1–4, pyruvate dehydrogenase kinases types 1–4; PDP 1,2, pyruvate dehydrogenase phosphatases types 1,2.

that IPG-P played a significant role in regulating glucose metabolism at the PDC stage by sigmoidal inhibition of PDK in addition to the linear activation of PDP. At critical concentrations of IPG-P an enhancement of the switchover mechanism to the active de-phospho form of PDC occurs. In the light of evidence for the release of IPGs from membrane preparations by insulin (15, 26–28), it is suggested that a short term effect of insulin on PDC may be mediated, in part, by the reciprocal control of PDP and PDK, an effective push-pull system.

EXPERIMENTAL PROCEDURES

Tissues and Reagents—Wistar albino male rats weighing 130–150 g, from Harlan Olac UK Ltd. (Bicester, Oxon, UK) were used for the preparation of the IPGs from liver. CoA, pyruvate, thiamine pyrophosphate, dithiothreitol, and activated charcoal were purchased from Sigma. AG1-X8 resin (OH^-, 20–50 mesh, converted to the formate form) was purchased from Bio-Rad. Salts, organic solvents, and other reagents were of analytical grade or better. PDC and PDP (metal-dependent form) were prepared from beef heart as described by Lilley *et al.* (29).

Preparation of IPGs—IPG-P and IPG-A extracted from 5 to 10 g of tissue were separated by elution from Amberlite columns (formate form) with HCl, pH 2.0 and pH 1.3, respectively, as described by Nestler *et al.* (30) and as modified by Caro *et al.* (16). The freeze-dried fractions were stored at −80 °C and for use were dissolved in distilled water. Further stages of purification of the IPG-P were as described in detail by Caro *et al.* (16). A parallelism between the activation of PDP and inhibition of PDK was observed at each stage of the purification procedure. The final product was subjected to paper chromatography and elution with butanol/water/ethanol at a ratio of 4:1:1. Fraction 1 contained on average 67% of the initial IPG-P activation of PDP and 58% of inhibition of PDK; <10% was found in fraction 2, and all other fractions (fractions 3–7) contained <5% of the original activity. On the basis of this evidence, there appeared to be no grounds for considering that the effectors of PDP and PDK were separate entities.

Synthetic Inositol Hexosamine Analogues—The following compounds were synthesized (31–37). Representative structures are shown in Figs. 1, 2, and 8: series 1, $GlcNH_2\alpha1-6$-D-*myo*-inositol (**1**) and $GlcNH_2\beta1-6$-D-*myo*-inositol (**2**); series 2, $GlcNH_2\alpha1-6$-D-*myo*-inositol-1(2)-*O*-PO_3 (**3**), $GlcNH_2\alpha1-6$-D-*myo*-inositol-5-*O*-PO_3 (**4**), $GlcNH_2\beta1-6$-D-*myo*-inositol-2(3)-*O*-PO_3 (**5**), and 3′*O*-methyl-$GlcNH_2\alpha1-6$-D-*myo*-inositol-3,4 di-*O*-PO_3 (**6**); series 3, 6-*O*-$PO_3GlcNH_2\alpha1-6$-D-*myo*-inositol (**7**), 4-*O*-PO_3-$GlcNH_2\alpha1-6$-D-*myo*-inositol (**8**), and 3-*O*-PO_3-$GlcNH_2\alpha1-6$-D-*myo*-inositol (**9**); series 4, $GlcNH_2\alpha1-6$-D-*myo*-inositol-5-*O*-acetate (**10**), $GlcNH_2\alpha1-5$-D-*myo*-inositol-6-*O*-acetate (**11**), and 2-azido-2-deoxy-3-*O*-benzyl-$GlcNH_2\alpha1-6$-D-*myo*-inositol-6-*O*-acetate (**12**); series 5, $GlcNH_2\beta1-6$-D-*chiro*-inositol (**13**), $GlcNH_2\alpha1-6$-D-*chiro*-inositol (**14**), and $GalNH_2\beta1-6$-$GalNH_2\beta1-1$-D-*chiro*-inositol (**15**); series 6, $GlcNH_2\alpha1-6$-D-*chiro*-inositol-1-*O*-PO_3 (**16**) and $GlcNH_2\beta1-6$-D-*chiro*-inositol-1(2)-*O*-PO_3 (**17**); series 7, $GalNH_2\beta1-6$-D-pinitol (**18**), $GalNH_2\beta1-4$-D-pinitol (**19**), $GalNH_2\beta1-4$-L-pinitol (**20**), $GlcNH_2\alpha1-6$-D-pinitol (**21**), $GlcNH_2\beta1-6$-D-pinitol(**22**), and $GalNH_2\alpha1-6$-D-pinitol (**23**).

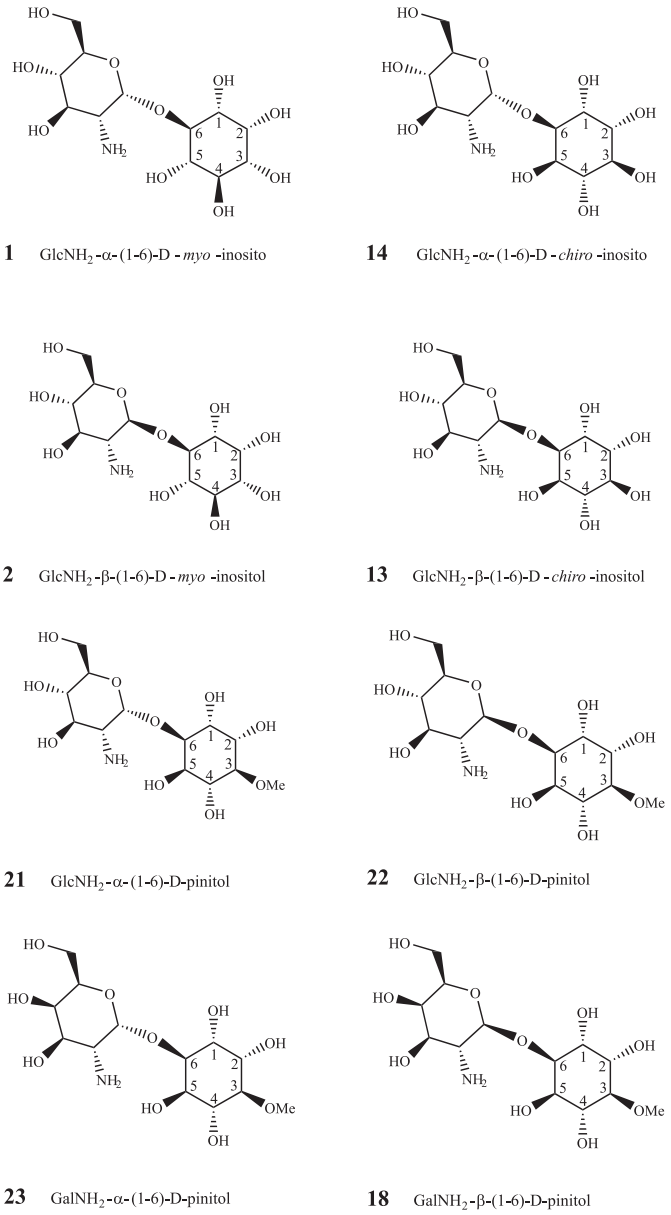
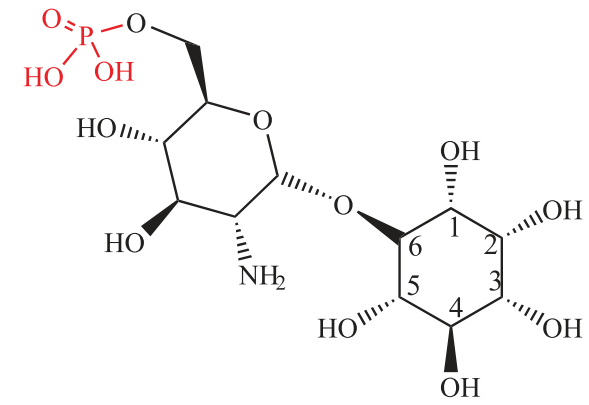


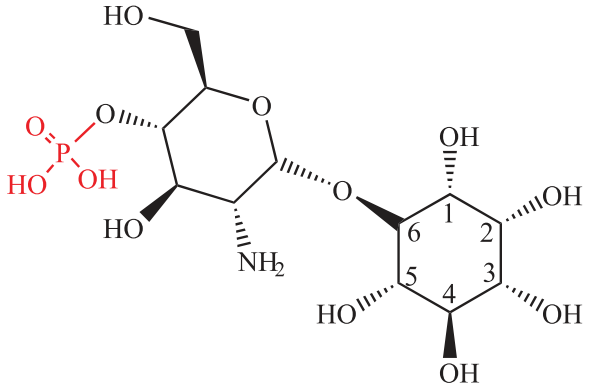
FIGURE 1. **Structures of representative synthetic nonphosphorylated hexosamine inositols.**

Measurement of IPG-P and IPG-A—The bioassays were as described previously (16, 23, 29). In brief, IPG-P was determined using the activation of PDP by the spectrophotometric variant of the two-stage system described by Lilley *et al.* (29). A unit of activity is defined as the conversion of 1 nmol of NAD^+ to NADH per min at 30 °C. IPG-A was assayed by the stimulation of incorporation of [U-^{14}C]glucose into lipids of adipocytes isolated from rat epididymal fat pads by the method of Rodbell (38). A unit of IPG-A is defined as 1 nmol of [^{14}C]glucose incorporated into lipid/g dry weight/h at 37 °C.

Preparation of Heart PDC and Assay of PDP and PDK—PDC was partially purified from beef heart mitochondria as described previously (29), and this complex contains a tightly associated PDK (2, 4, 8). The PDP was separated from PDC by washing the PDC three times with a buffer mixture containing the following: 20 mM potassium phosphate buffer, pH 7.0, 1 mM



7 6-O-PO_3 $GlcNH_2$ - α-(1-6)-D-*myo*-inositol



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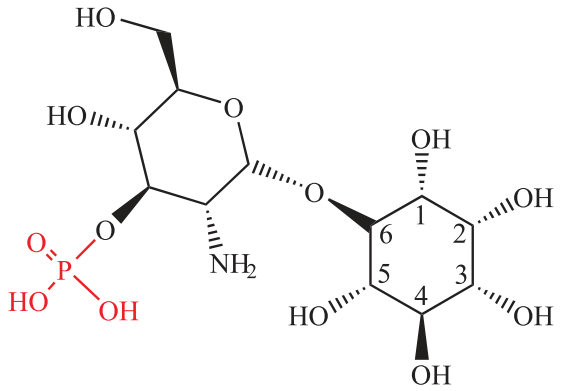

9 3-O-PO_3 -$GlcNH_2$ - α-(1-6)-D-*myo*-inositol

FIGURE 2. **Structures of the 6-phosphate, 4-phosphate, and 3-phosphate analogues of $GlcNH_2$α6-D-*myo*-inositol.**

dithiothreitol, 1 mM $MgCl_2$ (16). The specific activities of the heart preparations used were as follows: PDC, 1.4 units/mg protein and PDP 1.8 units/mg protein (1 unit produces 1 μmol of NADH/min/30 °C; these were stored at −80 °C until used). These preparations were free from lactic dehydrogenase, ATPase, and activity in the reoxidation of NADH.

Assay of PDP—The assay of PDP is based on the reactivation of the phosphorylated form of PDC by incubation with the separated soluble beef heart PDP component and measurement of the activity of PDC by the rate of reduction of NAD^+ using the two-step procedure of Lilley *et al.* (29) as previously described by Caro *et al.* (16). After inactivation with ATP the activity of PDC was reduced to less than 1% of the original value (see supplemental material).

Assay of PDK—The assay of PDK was based on the rate of inactivation of PDC by ATP catalyzed by the PDK component of the complex, a first order rate reaction that is basically similar to that described by Bajotto *et al.* (39). The active nonphosphorylated residual activity of PDC was measured spectrophotometrically (16). Full inactivation of PDC required 15 min (<1% of original activity), and the PDC was ~70% inactivated in 10 min. The amount of effector molecule being tested was adjusted so that the extent of inactivation was decreased from 70% with ATP alone to ~50% in the presence of the test sample. These parameters were set to achieve a response yielding linearity. The net effect of inhibition of PDK by IPG-P or synthetic analogues was a higher residual activity of PDC and faster rate of reduction of NAD^+ to NADH. The effects of IPG P-type from liver and of metal ions on the activity of PDC are presented as nanomoles of NADH/min at 30 °C or as percentage inhibition of PDK (see supplemental material).

Measurement of Free Ca^{2+} Ions—The ISM-146 Ca microcalcium electrode and the DJM-146 micro-reference electrode from Lazar Research Laboratories, Inc. were used. The calibration curve range was 0.02–6 mM $CaCl_2$ in an isotonic solution of NaCl (0.9%). The free Ca^{2+} in the PDP assay was 0.035 mM (mean of three values). The addition of Mn^{2+} (1.0 mM) did not alter the free Ca^{2+} over the range examined (0.01–1.0 mM).

Mn^{2+} and Zn^{2+} Content of IPG Preparations—Trace metals were measured in rat liver IPGs using an atomic absorption spectrometer (Philips PU 9100) with an air/acetylene flame; Mn^{2+} was measured at 279.5 nM and Zn^{2+} at 213.9 nM.

RESULTS

Effect of Liver IPG-P and IPG-A on PDC—The effects of IPG-P isolated from liver on PDP and PDK are shown in the Fig. 3, *a* and *b*. The sigmoidal dose-response curve contrasted sharply with the well established linear stimulatory effects of IPG-P types on activation of PDP (16–19, 29). The regulatory effect of the contrast between the linear activation of PDP and sigmoidal inhibition of PDK, the latter been manifest at higher concentration of IPG-P, is for a push-pull system that will magnify the response of PDC to moderate changes in IPG-P, a highly sensitive system at critical tissue concentrations of IPG-P. In contrast, IPG-A type from liver had no effect on PDK activity (data not shown).

Effect of Mn^{2+} and Zn^{2+} on PDK and PDP Activity—As shown in Fig. 4, *A* and *B*, the optimal concentration of Mn^{2+} (1 mM) was broadly similar for PDP and PDK contrasting with Zn^{2+}, which was 2 orders of magnitude lower than that for Mn^{2+} for PDP (0.01 mM) and an order lower for PDK (0.1 mM). These values are the total amounts in the assay systems and not the free, unbound concentrations. The possibility that Mn^{2+} might raise the free Ca^{2+} in the assay medium for PDP was tested using a calcium ion electrode; no significant change was detected. The Mn^{2+} and Zn^{2+} content of IPG-P and IPG-A

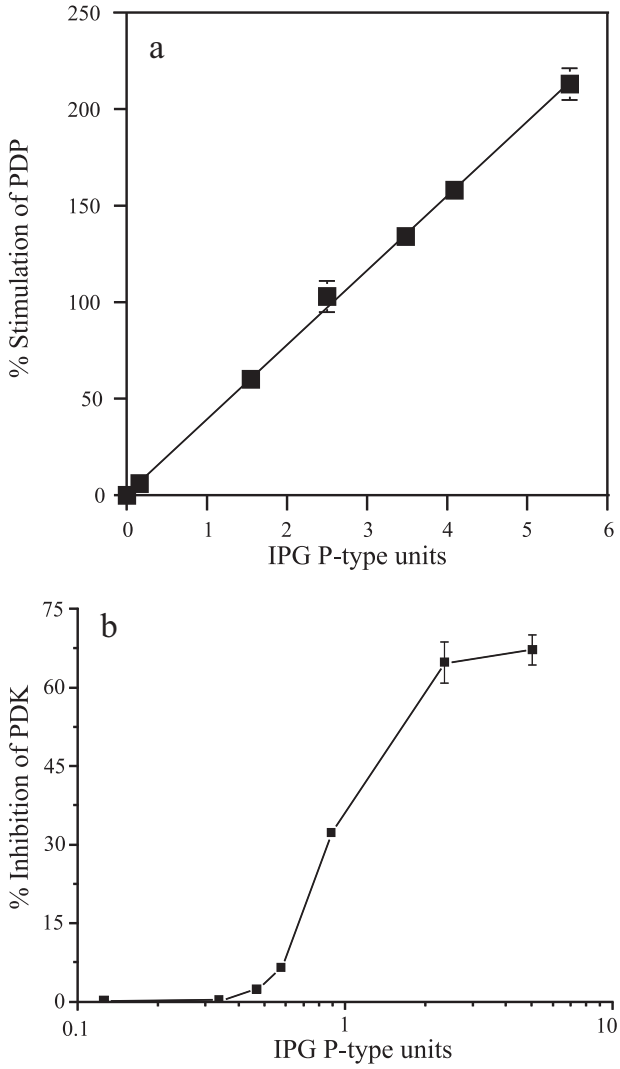

FIGURE 3. *a* and *b*, effect of IPG-P from rat liver on the stimulation of PDP and inhibition of PDK. *a*, linear relationship for the stimulation of PDP by IPG-P determined by the spectrophotometric measurement of the rate of reduction of NAD^+ to NADH; the results are the means ± S.E. of four separate estimations, and where no S.E. is given, the value is too small to show. An extended experiment revealed that linearity was maintained up to 350% stimulation (results not shown). *b*, percentage inhibition of PDK by IPG-P is shown in a positive direction because the greater the inhibition of PDK by IPG-P type the lesser the phosphorylation of PDC and conversion to the inactive phospho form. The active form of PDC was determined by the rate of reduction of NAD^+. The results are given as the means ± S.E. for six separate determinations, and where no S.E. values are given, they are too small to show; for details of assay systems, see "Experimental Procedures." Liver contained 8 ± 0.5 units of IPG-P per g (mean ± S.E. of six separate extractions). The assay mixtures contained 72 μg of PDC and/or 1.4 μg of PDP, both prepared from bovine heart.

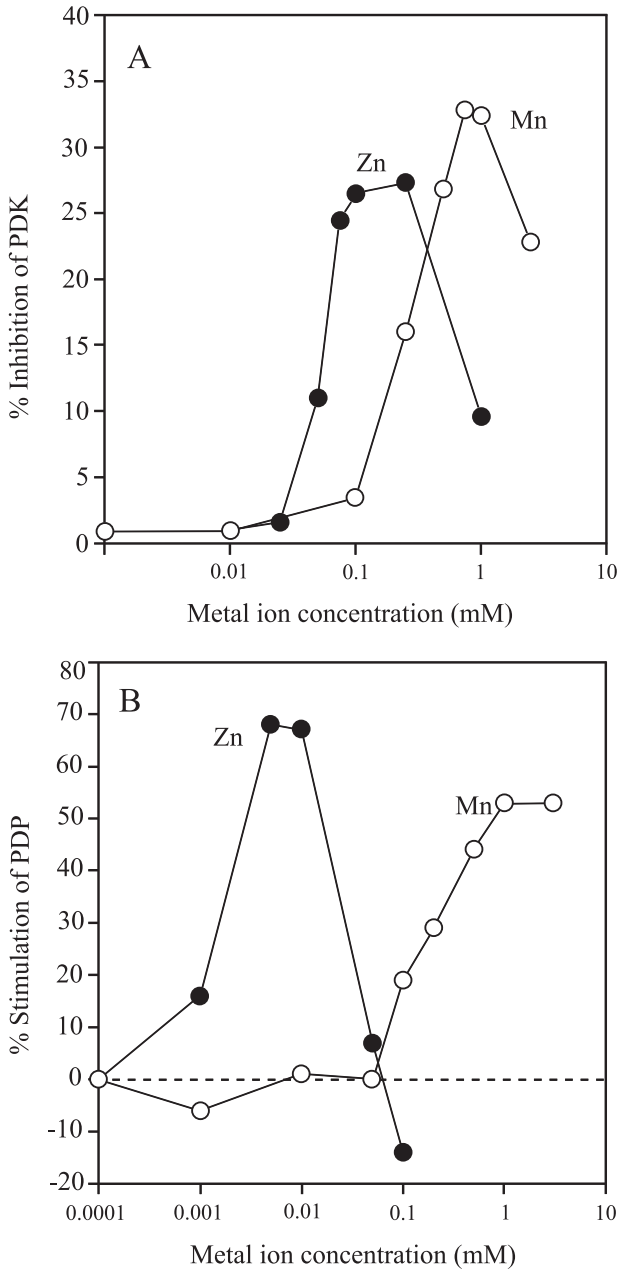

FIGURE 4. *A* and *B*, effects of Zn^{2+} and Mn^{2+} on the activity of bovine heart PDK and PDP. *A*, inhibitory effects of the trace metals on PDK. The percentage inhibition of PDK (using 72 μg of PDC, incubation time of 10 min) is shown in a positive direction because the greater the inhibition of PDK by Zn^{2+} and Mn^{2+}, the lesser the phosphorylation of PDC and conversion to the inactive phospho form. The active form of PDC is determined by the rate of reduction of NAD^+. The inhibition of PDK activity is a first-order reaction (see Ref. 39), and here linearity of response to trace metals was achieved by selecting a range of activity between 70 and 50% inhibition, see "Experimental Procedures." *B*, parallel experiments on activation of PDP (using 1.4 μg) by Zn^{2+} and Mn^{2+}; the metal ions values refer to the total and not the free concentrations. (Data in *B* are derived from Kunjara *et al.* (23) Fig. 8, with permission from Elsevier).

from rat liver measured by atomic absorption spectroscopy is given in Table 1, the Mn^{2+}/Zn^{2+} ratio was ~3:2. It has been shown that removal of metals with dithizone inactivated a liver IPG-P preparation, activity being partially restored by treatment with Mn^{2+} (16). The present experiments on the effect of dithizone on the IPG-P stimulation of PDP gave the following results: IPG-P, no treatment, activity +58%; dithizone treatment, activity +7%; 15 min with 2.7 mM Mn^{2+}, activity +30%; 15 min with 2.7 mM Mn^{2+} plus 1.8 mM Zn^{2+}, activity +45%.

Effect of IPG-P and Mn^{2+} on PDP—Significant differences exist between PDP1 and -2 in response to Ca^{2+} that is required for the interaction of PDP1 with the E2 component of PDC and stimulates PDP1, but which has no effect on PDP2 up to a concentration of 0.2 mM (1, 2, 4, 12). As shown in Fig. 5, *A* and *B*, in the absence of Ca^{2+}, Mn^{2+} and IPG-P increased the PDP activity by 2.7- and 7-fold, respectively, in combination an additive effect was observed (Fig. 5*A*). The addition of Ca^{2+} (0.1 mM)

TABLE 1

Mn^{2+} and Zn^{2} content of IPG-P and IPG-A from rat liver

IPG-P and IPG-A were extracted from rat liver (see "Experimental Procedures"). The fractions were dissolved to give a concentration of IPGs isolated from 1 g of liver in 1 ml of water. Estimations were in triplicate using two separate preparations of IPGs. The metals were measured by atomic absorption spectroscopy (see "Experimental Procedures").

	Mn^{2+}	Zn^{2+}	Mn^{2+}/Zn^{2+}
	μM	μM	
IPG-P (pH 2.0)	5.8, 7.7	2.8, 3.4	2.2
IPG-A (pH 1.3)	20.4, 24.9	15.0, 16.4	1.5

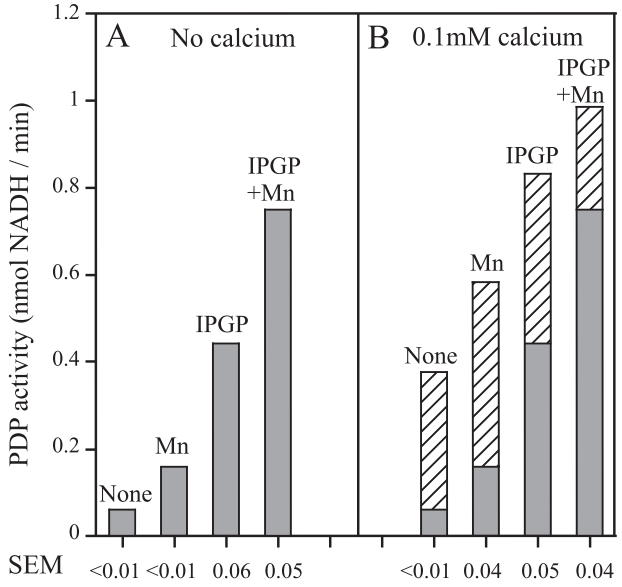

FIGURE 5. *A* and *B*, effect of Mn^{2+} and IPG-P alone or in combination on activation of bovine heart PDP in the presence and absence of Ca^{2+}. Effects of 1 mM Mn^{2+}, 4 units of IPG-P or Mn^{2+} plus IPG-P on PDP activity (using 1.4 μg of protein), (*A*) in absence of added Ca^{2+}, *solid bars,* or (*B*) in the presence of 0.1 mM Ca^{2+}, the additional effect of Ca^{2+} is shown by the *stacked hatched column*. S.E. values for not less than five separate experiments are shown at the *bottom* of each column.

alone gave a 6-fold increase in basal PDP activity; the incremental effect of Ca^{2+} appeared to be constant whether added alone, with Mn^{2+}, with IPG-P, or with Mn^{2+} plus IPG-P (Fig. 5*B*). It can be hypothesized that an effect of IPG-P and Mn^{2+} on bovine heart PDP might be on the PDP2 isoform, the added effect of Ca^{2+} being on PDP1. An action of IPG-P and Mn^{2+} on PDP1 cannot be excluded because the heart PDP preparation contained both isoforms, and Mn^{2+} can replace Mg^{2+} and Ca^{2+} (2). Measurements using the separate isoforms of PDP are required.

Activity of Synthetic Hexosamine Inositols—To date the only chemically characterized synthetic IPG is $GalNH_2\beta$4-D-pinitol (**19**) that has been termed INS-2. This compound was originally isolated from both rat and bovine liver (25). We were not able to confirm the activity of this compound as an allosteric activator of PDP either in the presence or absence of Mn^{2+}. We also synthesized the L form (**20**), and this was similarly inactive (data not shown). The nonphosphorylated analogues from the *myo* and *chiro* series (Series 1 and 5, see Fig. 1) were also found to have no activity as activators of PDP. In contrast α- and β-nonphosphorylated analogues of D-glucosaminyl and D-galactosaminyl 1–6 D-pinitol (Series 7) all had significant activity with the glucosaminyl derivative always more active than the corresponding galactosaminyl derivative (**21** *versus* **23** and **22** *versus* **18**). Because IPGs should contain phosphate groups, a series of hexosamine phosphate and inositol phosphate analogues was synthesized: Series 2, 3, and 6). These compounds were all more active than the nonphosphorylated glycosaminyl pinitols with clear structure-activity relationships. For example, comparison of the 6-*O*-phosphate-R (**7**) with (**8**) 4-*O*-phosphate-R and (**9**) 3-*O*-phosphate-R showed an activity (PDP) increase of 30, 125, and 325%, respectively (with R = $GlcNH_2\alpha$1–6-D-*myo*-inositol) as shown in Fig. 6*A*. Compounds **17** and **6**, which contained a phosphate(s) on the inositol, were also very active (PDP), 325 and 375% respectively. A number of acetate analogues were also synthesized (Series 4). Compounds **11** and **12** had significant activity in the PDP assay as shown in Fig. 6*C*. A number of the compounds also demonstrated activity as inhibitors of PDK (Fig. 6, *B* and *C*); compound **6** was the most active. This ability to both activate PDP and also inhibit PDK in a single structure was unexpected, and the result for compounds with both activities is shown in Fig. 6*C*.

DISCUSSION

It is widely held that the reversible phosphorylation of proteins regulates almost all aspects of cell life. One of the most widely studied signaling mechanisms in eukaryotic cells is the phosphorylation by protein kinases (5, 6). The regulation of PDC activity via isoform-specific acute regulation of PDKs and PDPs and the role of PDKs, in particular PDK4, acting as allostats in glucose homeostasis and lipid status have been reviewed (8). As proposed by Harris *et al.* (4), simultaneous opposing regulation of PDP and PDK could result in enhanced regulation of PDC. Evidence for such a dual control is supported by the work of Huang *et al.* (12) on the effects of starvation and diabetes on rat heart and kidney, the reported opposite changes in the expression of specific isoforms of PDP and PDK contributing to the hyperphosphorylation and the inactivation of PDC in these tissues. This concept is also illustrated by changes in heart PDC in starvation and diabetes (10). The increase in PDK4 and diminished expression of PDP1 in skeletal muscle of Otsuka Long Evans Tokushima Fatty rats before the onset of the genetically determined diabetes type 2 also indicated the importance of dual control of the kinase and phosphatase components of PDC (39). These hormonal and dietary factors center upon the changes in mRNA of specific isoforms of PDK and PDP and the expression of these proteins, properties that do not always go hand in hand (8, 11, 12). The coordination of changes in PDP and PDK and their low turnover number is also significant in relation to the preservation of ATP and the prevention of the action of the phospho/de-phospho reactions acting as a futile cycle (2).

Effect of IPGs on the Reciprocal Relationship between PDP and PDK—In contrast to the reciprocal regulation of the phospho/de-phospho cycle of PDC that has been established at the level of expression of the isoforms of PDK and PDP, there is at present scant evidence for regulatory factors acting *in vivo* in a reciprocal manner as "on-off" switch mechanism. As reviewed by Roche *et al.* (2), the metabolite shifts associated with pyruvate oxidation, namely AcCoA/CoA, NADH/NAD^+, ATP/

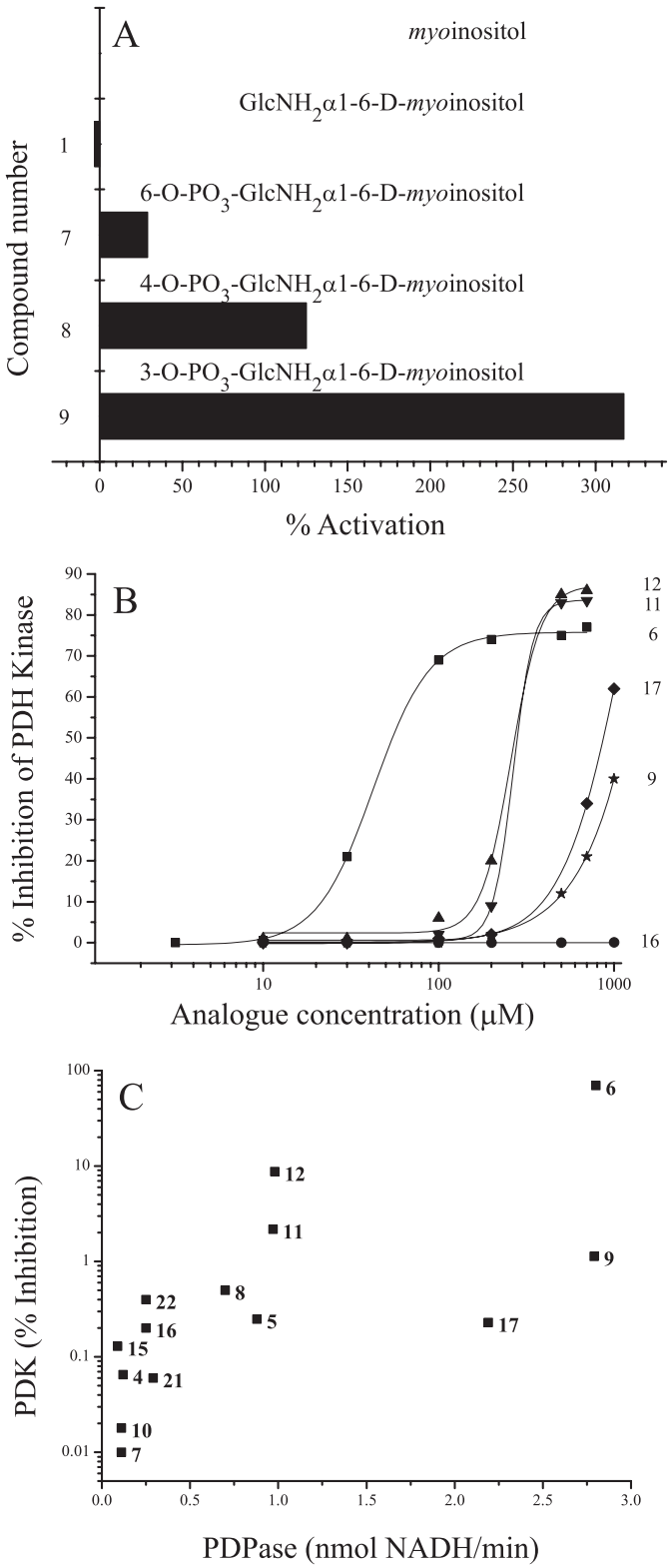

FIGURE 6. *A,* structure-activity relationship of phosphate analogues on PDP activity. Percent activation of PDP by 100 μM analogue. *B,* structure-activity relationship of analogues on PDK activity. Percent inhibition of PDK by various analogues was measure as a function of concentration. Series 5 analogues (acetate series) were more active than the mono-phosphorylated compounds. The di-phosphorylated compound (**6**) as the 3′-*O*-methyl derivative was the most active compound tested. *C,* comparison of activity of analogues that had activity on both PDK and PDH. Analogues were tested for activity at either 1 mM (for PDK see supplemental material) or 100 μM (for PDP see supplemental material).

ADP quotients, act specifically and differentially on the regulation of PDK isoforms; NADH was the sole product of the reaction for which there was a weak, and indeed sometimes undetectable, inhibition of PDP1 activity; hence the potential for the redox state of mitochondrial NADH/NAD^+ to regulate PDK and PDP is not well established (2). Thus, IPG-P appears to play a unique role in the reciprocal control of PDK and PDP demonstrated here in the *in vitro* systems using heart mitochondria, resetting the dynamic balance between the active/inactive forms of PDC. Multiple isoenzymic forms of PDK in different tissues raises the possibility that targeted responses could be elicited by specific IPGs (15, 19, 40, 41). The linear and sigmoidal effects of IPG-P on PDP and PDK add a significant degree of fine control, respectively, and this is further enhanced by the interaction of IPGs, IPG-A counteracting the effect of IPG-P activation of PDP (23).

Role of IPGs and Trace Metals in the Regulation of PDC—In view of the number of enzymes and compartmentation of biosynthetic pathways affected by IPGs and associated trace metals, the following factors need to be considered: (i) the free *versus* the total concentration of the metal ions; (ii) that PDP was in a soluble form and thus accessible to the effector molecules in contrast to PDK associated with the mitochondrial membrane; (iii) that the *in vitro* system used to study effects on the PDP and PDK is not subject to the complex intracellular network of metal trafficking pathways (42, 43) (Luk *et al.* (42) stated: "The current paradigm is that metal ions are not free agents. Rather, these ions are under careful surveillance by systems designed to detoxify sequester the metal or to escort the ion to its cognate site in a metalloprotein."); and (iv) differential effects of zinc (44, 45) and manganese (46–51) on acetyl CoA formation and utilization.

The data presented in Table 1 and Fig. 4, *A* and *B,* suggest that Zn^{2+} ions may be the more significant in the regulation of PDC. Although maximal activation of PDP is achieved with 0.01 μM Zn^{2+}, significant activation is observed at a concentration of 0.003 μM, a value commensurate with the measured content of zinc in IPG-P isolated from rat liver. Interestingly, this value is poised on the steeply rising slope of activation, and thus small changes in availability of Zn^{2+} could have significant effects on PDP activity. The higher level of Zn^{2+} required in the inhibition of PDK may relate to the association of the enzyme with the mitochondrial membrane and the requirement for a transport system or catalytic chelators to locate the metal ion at the active site. These differences in zinc ion concentration do not necessarily detract from the push-pull concept of the IPG-P in the control of PDC but rather to limitations of the *in vitro* system used.

The hyperbolic curve of the effect of Zn^{2+} on PDP (Fig. 4*B*) falling steeply at concentrations above 0.01 mM may be of significance in relation to the inhibitory effects of IPG-A on the activation of PDP by IPG-P reported previously (23). IPG-A has a 5-fold higher content of zinc relative to IPG-P, and it is suggested that this level of zinc might reach a concentration commensurate with those on the descending limb of the bell-shaped curve. In contrast to the regulation by Zn^{2+}, the effect of Mn^{2+} on the components of PDC is seen only at considerably higher concentrations (Fig. 4*A*).

Channeling of Acetyl CoA into Oxidative or Lipogenic Routes—The potent stimulatory effect of zinc on lipogenesis (52) is considered here in relation its effect on enzymes linked to the formation of acetyl CoA and direction of this metabolite toward lipid synthesis. It is postulated that central to this role of zinc is its action of resetting PDC to the active nonphosphorylated state thus promoting the formation of acetyl CoA. The effect of zinc on the activation of enolase in the glycolytic pathway (53) and the regulation of the phosphorylation state of the insulin receptor (54) further enhance the formation of acetyl CoA. The counter-effect of zinc in depressing acetyl CoA oxidation via inhibition of key enzymes of the tricarboxylic acid cycle, including aconitase, NAD-linked isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate oxidation, and cytochrome *c* oxidase (44, 45), could have the effect of both depressing ATP formation and preserving citrate for export to the cytosolic compartment thus driving acetyl CoA toward the lipogenesis. There is increasing interest in the insulinomimetic actions of zinc complexes on adipocytes *in vitro* (55) and use in the treatment of type 2 diabetes and metabolic syndromes (56).

In contrast, IPG-A contains a high content of manganese and is itself a known activator of lipogenesis and acetyl-CoA carboxylase (17). Scorpio and Masoro (50) have shown that the acetyl-CoA carboxylase system is highly sensitive to Mn^{2+}, and ~50% of maximal activation was shown at 25 μM Mn^{2+}, a concentration commensurate with the manganese content of IPG-A. In addition to activation of acetyl-CoA carboxylase by a manganese-dependent phosphatase (51), manganese activation of ATP-citrate lyase (57) and "malic" enzyme (58) contributes to lipogenesis, the latter by the provision of NADPH for reductive synthesis and anaplerotic provision of pyruvate. It may be noted that both zinc and manganese are involved in the activation of enolase (53). Additionally, manganese increases the stimulation by ATP of a putative insulin mediator from liver plasma cell membranes (48) and can largely overcome the regulatory feedback mechanisms of a high fat diet and increase lipogenic enzymes (44, 47).

IPG-P and Activation of PDP—The two isoforms of PDP are genetically and biochemically distinct, PDP1 requiring Ca^{2+} and PDP2 being insensitive to Ca^{2+} up to 0.2 mM (2). Here (see Fig. 5, *A* and *B*) the activation of the heart PDP preparation by IPG-P in the absence of Ca^{2+} indicated that IPGs might target the PDP2 isoform, consistent with the studies of Huang *et al.* (12) pointing to insulin status as regulating PDP2 mRNA and protein expression. The regulation of PDC activity via isoform-specific regulation of PDPs has been reviewed (59); PDP1 is regarded as a sensor of energy requirements where mitochondrial Ca^{2+} shifts occur, such as in increased workload and adrenergic stimulation in muscle and heart, whereas PDP2 responds to starvation and diabetes in rat heart and kidney (12). As reviewed by Roche *et al.* (2) and shown by Western blots (60), PDP2 is primarily found in fat-synthesizing tissues and is regarded as a target for activation by insulin.

Synthetic IPGs—A screen of 23 synthetic compounds resulted in the identification of a number of structures that demonstrated activity as both activators of PDP and inhibitors of PDK (Fig. 6*C*). For the natural IPG-P tissue-extracted material, this dual activity had been thought to indicate a family of compounds present in a mixture. The ability of single compounds to have this dual action was therefore unexpected. All of these compounds would therefore be expected to have a significant push-pull effect on the PDH complex. Fig. 7 compares three structures (Fig. 8) that had identical dose-dependent activity profiles and were indistinguishable in activity. All three of these compounds can be considered 6-substituted hexosamine analogues of the intracellular inositol polyphosphate mediators. For example structure (**6**) could be considered as IPG(3,4)P_2 by analogy with inositol 1,3,4-trisphosphate or phosphatidylinositol 3,4-bisphosphate. A larger series of structures clearly needs to be examined, but the current data indicate that IPGs may simply be hexosamine derivatives of the intracellular inositol polyphosphates. The inositol polyphosphates are produced intracellularly, do not activate or inhibit any of the known enzymes that are affected by the IPGs, nor do they freely cross or are translocated across cell membranes. In contrast their putative hexosamine derivatives are released from the external cell surface and transported back into the intracellular compartment where they act as allosteric activators and inhibitors of a large number of enzymes involved in metabolic actions. It is interesting to speculate that the hexosamine substitution is permissive of these activities.

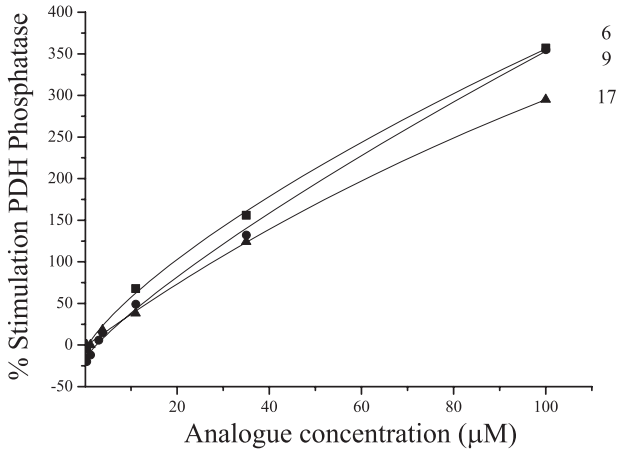

FIGURE 7. **Comparison of analogue activity in PDP assay.** Analogues at various concentration were tested for stimulation of PDP. A linear dose dependence was observed up to 100 μM.

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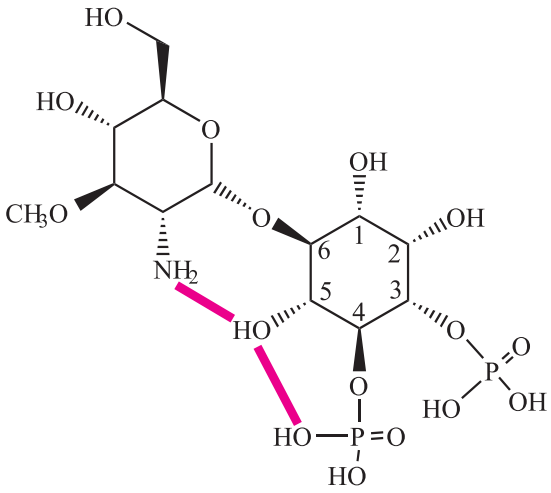
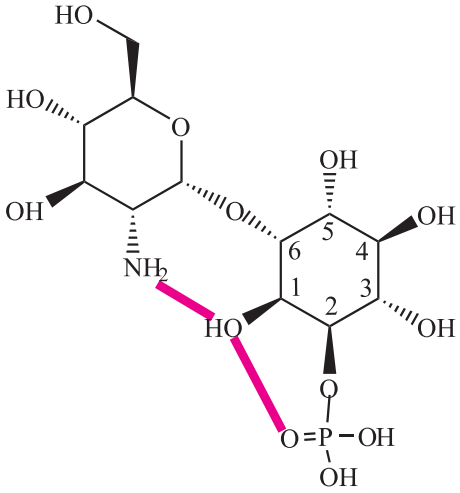
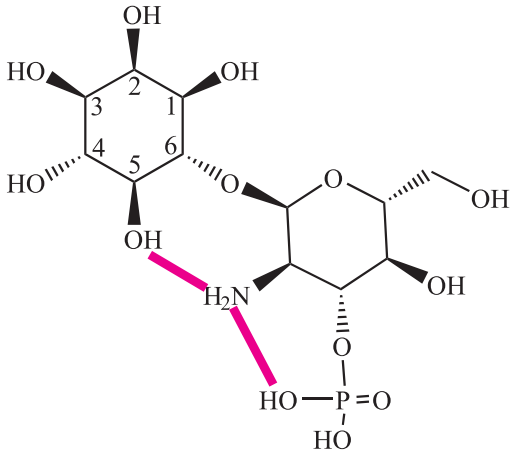
Addendum—That IPGs are of fundamental importance in cellular regulation is attested by the conservation of the signaling system from yeast to eukaryotes (15, 18, 61). Müller *et al.* (61) have shown that phosphoinositol glycan peptides from yeast potently induce metabolic insulin actions in isolated adipocytes and, furthermore, stimulated glucose transport in isolated cardiomyocytes, and glycogenesis and glycogen synthase in isolated rat diaphragms. The concentration-dependent effects reached 70–90% of the maximal insulin effect activity at low concentrations of the mediator (EC_{50} values 0.5–5 μM). More recently, modulation of protein phosphatase 2A, protein kinase A, and cAMP phosphodiesterase has been described (62), together with the translocation of glycosylphosphatidylinositol-anchored proteins from plasma membranes to lipid



6 3'-O-Methyl-GlcNH$_2$-α-(1-6)-D-myo-inositol 3, 4-di-O-phosphate



17 GlcNH$_2$-β-(1-6)-D-chiro-inositol-1(2)-PO$_3$



9 3-O-PO$_3$-GlcNH$_2$-α-(1-6)-D-myo-inositol

FIGURE 8. **Structures of compounds that had identical activity concentration curves for activation of PDP.** Spatial analysis indicates they may all have a common pharmacophore that is indicated by the *solid line*. Compound (**17**) was synthesized as the inositol 1-phosphate; however, cis migration to the 2-position can potentially occur.

droplets in rat adipocytes (63). Brautigan (64) stressed the importance of constant cycling as a background to rapid hormone response; with constant cycling the concentration of second messengers can be poised near the thresholds for activation. A "switchover" mechanism is reported here in the critical concentration at which the sigmoidal response of the inhibition of the PDK by IPG-P is brought into play amplifying the activation of PDC by PDP. There is a requirement for the termination of a signal because sustained signaling in response to an effector molecule is normally undesirable and, in the case of protein kinases, can be the basis of a number of disease states (5, 6). In this context, the interaction of IPG-P and IPG-A in the regulation of PDP suggests that the balance between the two families of IPGs may be one component of signal modulation (23) in addition to their enzymic cleavage (14, 16).

Recent reviews have emphasized that abnormal phosphorylation is causal in many diseases; some 19 have been linked to mutations of particular protein kinases and phosphatases (see Refs. 5, 6). Thus, specific protein kinase inhibitors for treatment of diseases, including diabetes, are high on the pharmaceutical agenda (65). PDK4 is selectively up-regulated in most tissues in response to starvation, diabetes, and insulin-resistant states, whereas parallel increases in the expression of the PDK4 and PDK2 isoforms are most apparent in gluconeogenic tissues (66). Dichloroacetate (DCA), a nonspecific PDK inhibitor, has been shown to increase both muscle and liver PDC activity and to reduce the blood glucose level in rat models of diabetes. The inhibition of PDK by DCA has been shown to be a potential treatment in a number of clinical conditions. Bonnet *et al.* (67) have shown that DCA shifts cancer cell metabolism from aerobic glycolysis to oxidative metabolism via a mechanism involving inhibition of PDK, decreased mitochondrial membrane potential, increased mitochondrial H_2O_2 production, activation of Kv channels, and induction of apoptosis and decreased proliferation without apparent toxicity to normal cells. The importance of PDK2 was further established by their report that inhibition of this isoform by short interfering RNA mimicked the action of DCA (67). Positive effects of DCA on the restoration of generation of ATP have been reported in the treatment of short periods of ischemia in heart (68) and in cerebral ischemia therapeutics (69). However, the long term use in chronic ischemic conditions may be precluded by potential toxic side effects. The search is on for small molecular weight high specificity inhibitors of PDK (70, 71). The compound AZD7545 is an inhibitor of the PDK2 isoform and has been shown to activate PDH *in vivo* and improve blood glucose control in obese (fa/fa) Zucker rats (70). The function IPG-P as an inhibitor of PDKs is the main focus of the present experiments. In view of the dual push-pull effect of IPG-P in activating PDP and inhibiting PDK, analogues of IPG-P may prove to be promising candidates in the treatment of a range of conditions, including diabetes (72).

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