Biochemical and Biophysical Research Communications **255,** 150–156 (1999)
Article ID bbrc.1999.0111, available online at http://www.idealibrary.com on IDEAL®

A Model Phosphatase 2C → Phosphatase 1 Activation Cascade via Dual Control of Inhibitor-1 (INH-1) and DARPP-32 Dephosphorylation by Two Inositol Glycan Putative Insulin Mediators from Beef Liver

L. C. Huang, D. Heimark, J. Linko, R. Nolan,* and J. Larner
Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908; and
**Insmed Pharmaceuticals, Inc., 800 E. Leigh Street, Richmond, Virginia 23219*

Received December 18, 1998

Two inositol phosphoglycans (IPG) isolated from beef liver and designated as putative insulin mediators were demonstrated to reciprocally enhance the dephosphorylation of inhibitor-1 (INH-1) and DARPP-32, thus directly activating phosphatase 2C and disinhibiting phosphatase 1 in a potential protein phosphatase 2C → phosphatase 1 cascade mechanism. One IPG termed pH 2.0, containing Dchiro-inositol and galactosamine, stimulated the dephosphorylation of INH-1 and DARPP-32 in a dose-dependent manner in the low micromolar range. A second, termed pH 1.3, containing myo-inositol glucosamine and mannose acted reciprocally to inhibit the cAMP-dependent protein kinase phosphorylation of INH-1 and DARPP-32 in a dose-dependent manner in the low micromolar range. These model experiments are discussed in terms of the observed dephosphorylation of INH-1 with insulin action documented in the literature and the activation of both phosphatase 1 and 2C described in intact cells and *in vivo* with insulin action. © 1999 Academic Press

Following up on the early work of Hizukuri and Larner demonstrating a Mg^{++} + $S0_3$ = activated conversion of rat liver glycogen synthase D to form I, (1), Bishop and coworkers (2) importantly demonstrated a metal (Mg^{++}, Mn^{++}) requirement for the dephosphorylation and activation of glycogen synthase in EDTA- and fluoride-treated ("demetallated") phosphatase preparations. Tsuiki *et al.* next purified a glycogen synthase "specific" phosphatase from rat liver, inactive on phosphorylase, which required metal, Mg^{++} or Mn^{++}, for activity (3). Tamura *et al.* subsequently cloned the enzyme, expressed it in *E. coli* and termed it 2C, since by sequence analysis, it proved unrelated to phosphatases 1,2A and 2B (4). Subsequent cloning of PDH phosphatase catalytic subunit by L. Reed and coworkers (5) demonstrated 22% amino acid identity and both enzymes are now recognized as members of a separate phosphatase family termed PPM (6). Recent cloning has established α, β(1–5) and γ isoforms of phosphatase 2C (7). The x-ray crystal structure of the α isoform containing two Mn^{++} coordination sites has been determined (8). Substrates preferentially dephosphorylated by phosphatase 2C have been identified and include AMP-dependent protein kinase (9) and CAM kinase II (10). From these studies a sequence incorporating a sensitive threonine P site with upstream N-terminal basic amino acids has been deduced. Studies with model peptides including RRATPVA again demonstrate a clear preference for threonine phosphate over serine phosphate (11).

Both INH-1 and DARPP-32 have a single threonine with basic residues on the N-terminal side. Thus, the sequences in INH-1 (RRRRPTPA) and in DARPP-32 (RRRPTPAML) are almost identical and both are potential substrates for phosphatase 2C. Both are known potent inhibitors of PP1 only when phosphorylated. Recent evidence has shown that both PP1 and PP2C are both activated with insulin action (12,13,14). A model mechanism to explain the activation of the two phosphatases via the allosteric actions of two inositol glycan (IPG) putative insulin mediators to promote the dephosphorylation of INH-1 and DARPP-32 and thus activate phosphatase 1 subsequent to activating phosphatase 2C, is demonstrated in the present experiments.

MATERIALS AND METHODS

Preparation of putative mediators. The pH 2.0 and 1.3 putative mediators were isolated from bovine liver as previously described (15). Typically, one kilogram of frozen liver was homogenized in 50

This work was supported by grants from Insmed Pharmaceuticals, Inc., Richmond, VA. and The Center of Innovative Technology, Commonwealth of Virginia.

0006-291X/99 $30.00

mM formic acid, 1 mM EDTA and 1 mM mercaptoethanol. After boiling and charcoal treatment, the clear homogenate was adjusted to pH 6.0, diluted with water and 700 ml of AG1 ×8 (formate form) added. After standing overnight with occasional stirring, the resin was packed into a column, the column was washed with water and then eluted with 0.015M HC1 (pH 2.0) followed by 0.1N HCl (pH 1.3).

The pH 2.0 eluate was then further purified by chelex column chromatography eluting with 0.1-0.2 M HCl to remove excess metals, silica gel column chromatography eluting with pyridine:propanol: acetic acid:water (8:8:1:4) to remove contaminating peptides and gel filtration over a column of Bio-gel P-4 to achieve further purification (TLC-single spot purity). The putative mediator activity was monitored by its stimulatory effect on pyruvate dehydrogenase phosphatase. The quantity of putative mediator was determined by its D-chiro-inositol content after hydrolysis in 6N HCl for 48 h. D-chiro-inositol was estimated by Dionex HPLC chromatography as described (16). Both IPGs were sensitive to HNO_2 deamination, and neutralized with selective antibodies.

The pH 1.3 eluate was first neutralized to pH 5 with NaOH, and then rotary evaporated to a small volume (4-5 ml). After desalting over a Sephadex G-10 column (2.6 cm × 85 cm), the aqueous eluate was passed through a C-18 column (2 cm × 14 cm) to remove contaminating peptides. The column pass-through and water wash were combined and lyophilized to dryness. The residue was dissolved in 1.0 ml H_2O and applied onto a Bio-gel P-4 column (1.6 cm × 85 cm) equilibrated with water. Fractions of 25 drops were collected and inhibitory effects on cAMP-dependent protein kinase were assayed. The inhibitory fractions were combined and lyophilized. The quantity of the putative mediator was determined by its myo-inositol content after hydrolysis in 6 N HCl as described (16).

Phosphorylation of INH-1 and DARPP-32 by cAMP-dependent protein kinase. 20 μl of INH-1 (1.6 mg/ml) kindly supplied by Dr. Shenolikar or DARPP-32 (2.2 mg/ml) kindly supplied by Dr. Nairn was incubated with [^{32}P]ATP, catalytic subunit of cAMP-dependent protein kinase (from Promega 200 μ), 10 mM $MgCl_2$, and 50 mM MES buffer, pH 7.1, at 37°C for 2 h. The unreacted ATP was removed by chromatography on a Sephadex G-25 column (1 cm × 60 cm) equilibrated with 20 mM imidazole, pH 7.4, containing 10% glycerol. The protein concentration in the [^{32}P]-labeled inhibitor fraction was determined by the modified method of Lowry (17).

Assay of phosphatase 2C activity. Phosphatase 2Cα was expressed in *E coli* and kindly supplied by Dr. Roger Nolan at Insmed Pharmaceuticals, Richmond, Va. and purified by the method of Tamura *et al*. (4) using the plasmid kindly provided by Dr. Tamura. To assay the effects of putative mediator, phosphatase 2C (0.05 μg), 20 mM imidazole, pH 7.4, 1 mM DTT, 0.5 mg BSA and indicated amounts of putative mediator were incubated at 37°C with [^{32}P]-INH-1 or [^{32}P]-DARPP-32 in a total volume of 100 μl. After 10 min of incubation, the reaction was terminated by the addition of 100 μl 50% TCA and 50 μl 5 mg/ml BSA. The precipitated protein was removed by centrifugation, and aliquots of the supernatant fluids counted.

Assay of cAMP-dependent protein kinase activity. cAMP-dependent protein kinase (type 1) was isolated from rabbit skeletal muscle as described (18). To assay the effects of putative mediator on the kinase activity, histone IIa, INH-1 or DARPP-32, MES buffer, pH 7.0, 300 mM; $MgCl_2$, 10 mM; cAMP, 1 μM; [^{32}P]ATP, 0.03 mM and pH 1.3 putative mediator as indicated were incubated with cAMP-dependent protein kinase (3μg) in a total volume of 100μl. Reactions were started by the addition of enzyme and incubations were carried out for 10 min at 30°C. Reactions were terminated by spotting 75μl aliquots of the reaction mixtures on to pieces of Whatman 3MM paper which were then dropped in 10% TCA and washed as described earlier (19). All assays were done in duplicate and experiments were performed at least twice to assure reproducibility.

RESULTS

Effects of pH 2.0 putative mediator on the dephosphorylation of INH-1 and DARPP-32. Fig. 1A demonstrates the dose dependence of added pH 2.0 putative mediator to stimulate the dephosphorylation of [^{32}P] INH-1 in the low μmolar dose range. At a substrate concentration of 0.3 μM, a 50 fold stimulation was observed. It is important to note that a thirteen fold stimulation was observed in the presence of saturating 10 mM $MgCl_2$ (Fig. 1A). Thus the pH 2.0 putative mediator exerts a stimulatory effect over the range of magnesium concentrations indicating that the stimulatory action of the putative mediator occurs in the presence of maximal metal ion. It is also of interest that [^{32}P] DARPP-32 (Fig. 1B) phosphorylated by the cAMP-dependent protein kinase is also dephosphorylated by PP2C. Importantly this dephosphorylation is also stimulated by the pH 2.0 putative mediator in a dose-dependent manner in the low μmolar dose range. The extent of the stimulatory effect of the putative mediator was similar with both INH-1 and DARPP-32. It was recently reported that dephosphorylation of serine 137 on DARPP-32 phosphorylated by casein kinase 2 is also dephosphorylated by PP2C (20). Here, we demonstrate that in the presence of pH 2.0 putative mediator the site phosphorylated by the cAMP-dependent protein kinase (presumably threonine 34) is also efficiently dephosphorylated by PP2C.

Effects of magnesium ion on the activity of PP2C. It is well established that PP2C is a magnesium (and manganese)-dependent phosphatase (3). Fig. 2 again demonstrates that in the absence of putative mediator, Mg^{++} stimulates the dephosphorylation of [^{32}P] INH-1 by PP2C with a maximal effect at about 3 mM. In the presence of 24 pmoles (determined as D-chiro-inositol after acid hydrolysis) of the pH 2.0 putative mediator, the enzyme is fully activated in the absence of Mg^{++}. In fact, in the presence of the putative pH 2.0 mediator, 10 mM Mg^{++} is slightly inhibitory. The same stimulatory effect of the pH 2.0 putative mediator in the presence of increasing Mg^{++} concentrations is observed using [^{32}P] DARPP-32 as substrate (data not shown).

Previously we reported that the pH 2.0 putative mediator leftshifted and upshifted the Mg^{++} dose–response curve of pyruvate dehydrogenase phosphatase acting on myosin light chains (21). In addition, we showed that the putative mediator abolished the Mg^{++} requirement of PP2C when phosphocasein was used as substrate phosphorylated by casein kinase II (unpublished experiments). Thus the action of this putative mediator on PDH phosphatase and on phosphatase PP2C to sensitize the enzyme to Mg^{++} appears to be a general phenomenon independent of substrate.

Effects of pH 1.3 putative mediator on the phosphorylation of INH-1 and DARPP-32. We next examined the action of the pH 1.3 putative mediator on the phos-

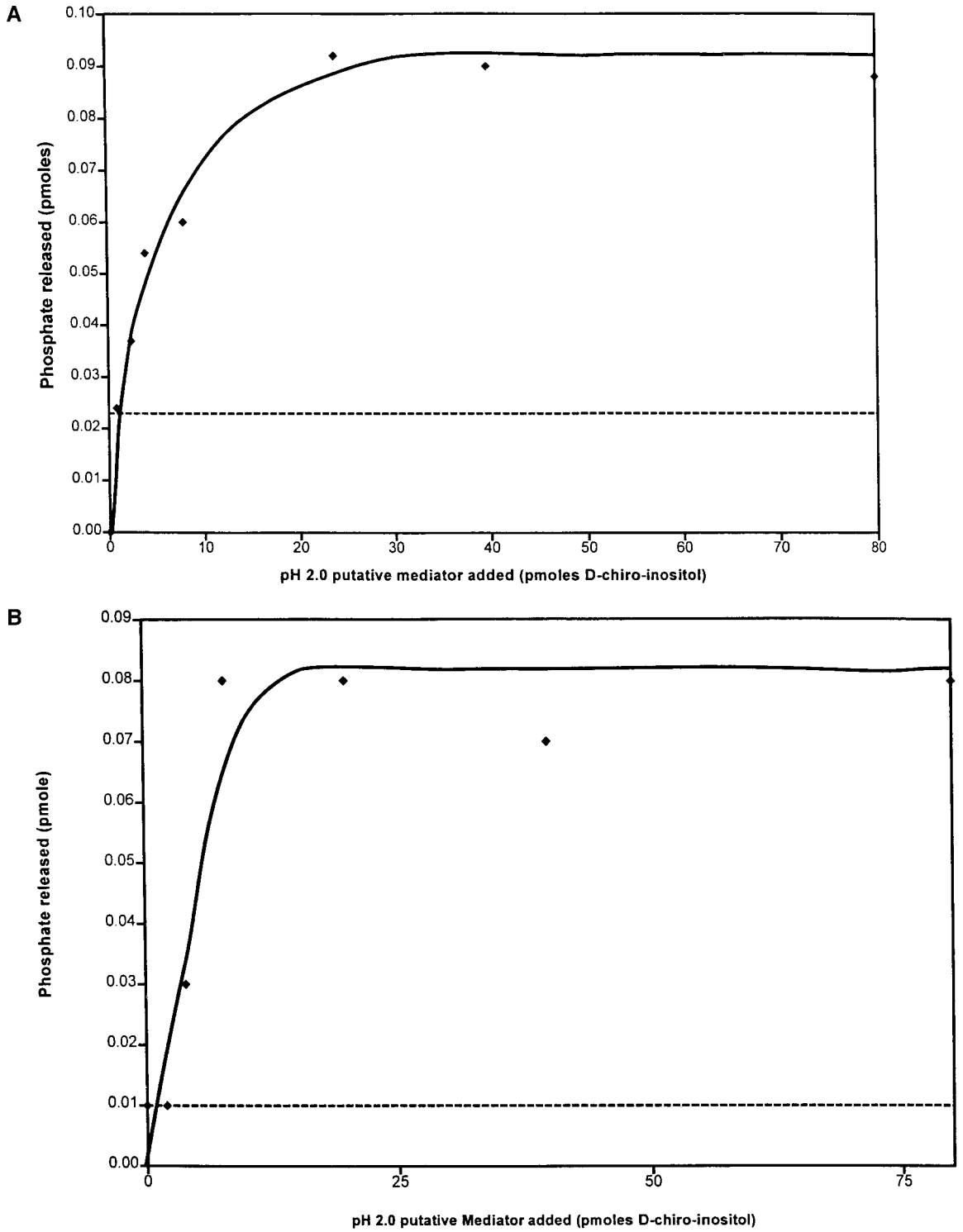

FIG. 1. A. Effect of pH 2.0 inositol glycan putative mediator on the dephosphorylation of inhibitor 1 (INH-1) catalyzed by PP2C. INH-1 was phosphorylated by cAMP-dependent protein kinase with [^{32}P]ATP. PP2C was incubated with [^{32}P] INH-1 under conditions described in the text. The pH 2.0 putative mediator was added as indicated. Putative mediator added, ◆. 10 mM $MgCl_2$ added, - - -. B. Effect of pH 2.0 inositol glycan putative mediator on the dephosphorylation of DARPP-32 catalyzed by PP2C. DARPP-32 was phosphorylated by cAMP-dependent protein kinase with [^{32}P]ATP. PP2C was incubated with DARPP-32 under conditions described in the text. pH 2.0 putative mediator was added as indicated. Putative mediator added, ◆. 10 mM $MgCl_2$ added, - - - .

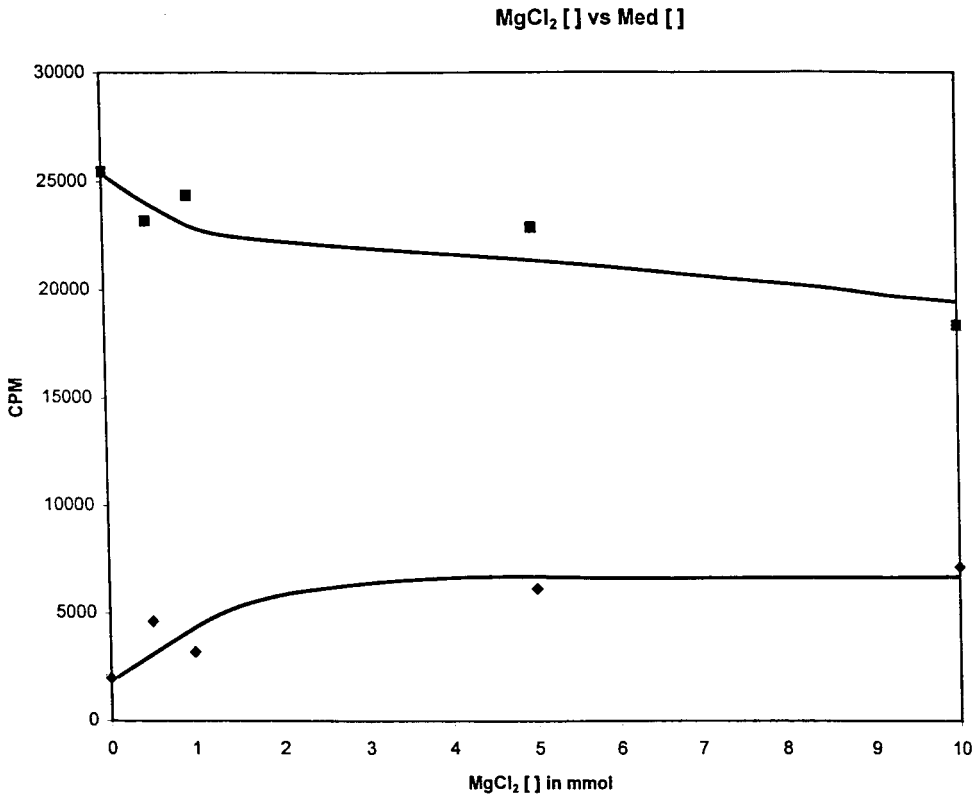

FIG. 2. Effect of $MgCl_2$ on the stimulatory action of pH 2.0 inositol glycan putative mediator. [^{32}P] INH-1 was incubated with PP2C under conditions described in the text except that the $MgCl_2$ concentrations were as indicated. No putative mediator was added, ◆. 24 pmole of pH 2.0 putative mediator was added, ■.

phorylation of INH-1 and DARPP-32 by the cAMP-kinase. The pH 1.3 putative mediator has been shown to inhibit cAMP-dependent protein kinase using histone IIa as substrate (22). Fig. 3A demonstrates that with INH-1 as substrate, inhibition of phosphorylation of INH-1 was dose-dependent and essentially complete in the presence of added pH 1.3 putative mediator at low μM concentrations. Unlike the experiments with PP2C and the pH 2.0 putative mediator, (Fig. 3) this inhibition was not affected by the Mg^{++} concentration, (data not shown). Fig. 3B also demonstrates that the phosphorylation of DARPP-32 by the cAMP-dependent kinase is again inhibited by the pH 1.3 putative mediator to the extent of 70% in a dose-dependent manner at the low μmolar range. Since DARPP-32 was phosphorylated to a lesser extent than INH-1 (25%), the efficacy of the pH 1.3 putative mediator to inhibit the phosphorylation was somewhat decreased over that observed with INH-1. In fact, the same inhibitory efficacy of the pH 1.3 putative mediator was also observed with histone as substrate (data not shown) and (22). Thus, we conclude that the pH 1.3 putative mediator acts by blocking the phosphorylation reaction catalyzed by cAMP-dependent protein kinase independent of substrate.

DISCUSSION

Evidence for at least five regulatory inputs involving specific kinase inhibition and phosphatase activation to explain insulin-stimulated glycogen synthase multisite (9 sites) dephosphorylation has appeared (23,24,12). (A) Insulin mediated cAMP-kinase inhibition via maintenance as a holoenzyme was initially demonstrated in sensitive tissues including muscle, liver and fat by several laboratories thus potentially explaining the dephosphorylation of sites 1, 2A and 2B (25,26,27,28). Most recently, (B) inhibition of GSK3 via covalent phosphorylation by PKB with upstream phosphorylation by PDK activated by P13K was elegantly demonstrated, potentially explaining dephosphorylation of sites 3A, B and C (29). Protein phosphatase 1 (PP1) (C) and most recently PP2C (D) have both been shown to be activated by insulin in adipocytes (12,13) and liver (14), with PP2C activation most recently demonstrated in liver biopsy samples of monkeys during an *in vivo* insulin infusion clamp procedure (14). In rat adipocytes (E) two separate pathways of glycogen synthase activation in the absence of glucose in the medium and in its presence were originally demonstrated (30). In the presence of glucose, a requirement

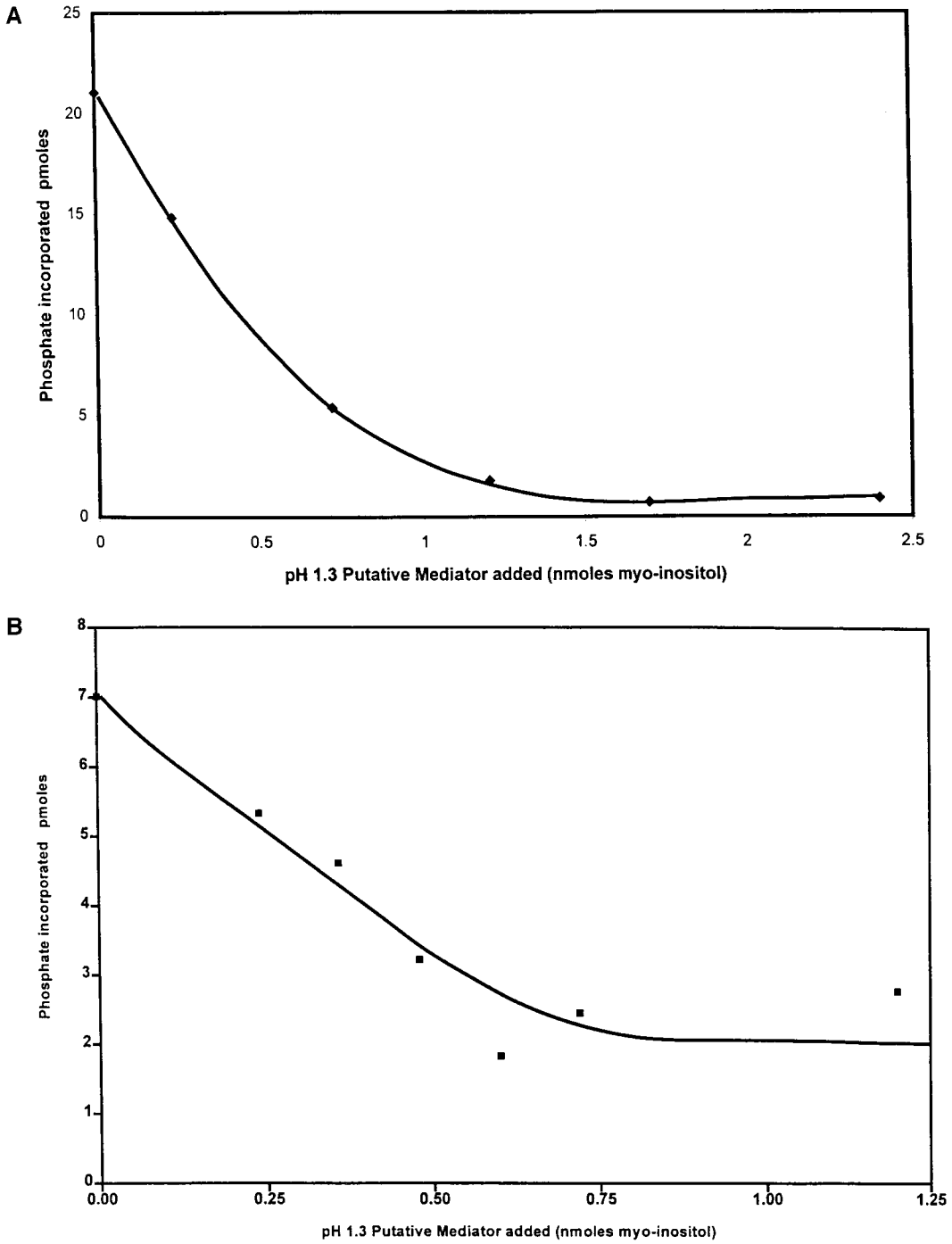

FIG. 3. A. Effect of pH 1.3 inositol glycan putative mediator on the phosphorylation of INH-1 catalyzed by cAMP-dependent protein kinase. INH-1 was incubated with cAMP-dependent protein and [^{32}P]ATP as described in the text. pH 1.3 putative mediator was added as indicated. B. Effect of pH 1.3 inositol glycan putative mediator on the phosphorylation of DARPP-32 catalyzed by cAMP-dependent protein kinase. DARPP-32 was incubated with cAMP-dependent protein kinase and [^{32}P]ATP under conditions described in the text. pH 1.3 putative mediator was added as indicated.

for both a hexose and its phosphorylation on the 6 position (i.e., glucose 6-P) was shown to be required for GS activation by insulin. The actions of glucose-6-phosphate have been reviewed recently by Villar-Palasi and Guinovart (31). In the absence of glucose, a separate mechanism and mediator was proposed (32,33).

We have separated, purified and partially determined the structures of two inositol glycan (IPG) putative mediators of insulin action (33). They were iden-

tified by their separate actions to stimulate Mg^{++} and Mn^{++} sensitive PDH phosphatase and phosphatase 2C in a kinetic manner similar to insulin (21) and to inhibit cAMP-kinase and adenylate cyclase (22). The former termed pH 2.0 (pH of elution from anion exchange column) was shown to be composed of D-chiro-inositol and galactosamine (33), and the latter termed pH 1.3 to contain myo-inositol-P, glucosamine and mannose (33). The complete structures will be the subject of future publications. They both are released with insulin *in vitro* from cell and cell membrane precursor lipids and/or proteins (34,35) and are also released in muscle tissue (36) and into the circulation with insulin action *in vivo* in humans (37). When injected *in vivo* they reduce hyperglycemia in STZ diabetic rats in a dose-dependent manner at low nanomolar doses comparable to insulin (38,15). The evidence for their consideration as putative mediators of insulin action has been summarized (39). To explain the activations of both phosphatase 1 and 2C with insulin observed in the literature (12,13,14) we studied the actions of both putative mediators to control the phosphorylation state of inhibitor-1 (INH-1) and DARPP-32.

In the present experiments we determined the effect of the pH 1.3 putative mediator to inhibit the action of cAMP-kinase to phosphorylate both INH-1 and DARPP-32. From the literature it is clear that under these conditions only the sensitive threonine in both inhibitors is phosphorylated (40,41). We further studied the effect of the pH 2.0 putative mediator to stimulate the dephosphorylation by phosphatase 2C of INH-1 and DARPP-32 phosphorylated by the cAMP-kinase. A mutually reinforcing dual action of both the pH 2.0 inositol glycan to stimulate phosphatase 2C to dephosphorylate and of the pH 1.3 inositol glycan to inhibit the phosphorylation by cAMP-kinase was shown, thus leading to a dual allosteric control of the dephosphorylation of both INH-1 and DARPP-32 in a potential phosphatase 2C $\rightarrow$ phosphatase 1 activation cascade mechanism. This mechanism is discussd in terms of the previously observed dephosphorylation of INH-1 with insulin in fat and muscle in the literature (42,43).

PP2C is clearly activated in a dose-dependent manner by the pH 2.0 putative mediator in the low μmolar dose range, to dephosphorylate INH-1 (Fig. 1A) and DARPP-32 (Fig. 1B). This occurs via a sensitization i.e., a leftshifting and upshifting of the enzyme's dose-response to Mg^{++} (Fig. 2). Similar results have been previously reported from this laboratory with PP2C and other substrates and with PDH phosphatase dephosphorylating PDH and myosin light chains (21). Thus the kinetic effect of the pH 2.0 putative mediator appears independent of substrate. Of considerable physiological importance are the experiments of Denton and coworkers (44) whose careful studies have documented a similar kinetic effect of insulin itself acting on fat segments. Following insulin action on fat segments, the Mg^{++} dose response of PDH phosphatase was also left-shifted (44).

As shown in Fig. 3A and 3B, the phosphorylation of INH-1 and DARPP-32 are both strongly inhibited by the pH 1.3 IPG putative mediator in a dose-dependent manner again in the low μmolar dose range. As previously shown, both pH 2.0 and pH 1.3 putative mediators are increased with insulin action *in vitro* (34,35) and *in vivo* in muscle biopsy samples during an insulin clamp procedure (36). Further, both putative mediators lowered elevated hyperglycemia in STZ diabetic rats in the low nanomolar dose range (15,38), and stimulate [^{14}C]glucose incorporation into diaphragm in normal rats similar to insulin (38).

Since this is an *in vitro* model set of experiments, it is of interest to review earlier literature reports that demonstrated that INH-1 is in fact dephosphorylated with insulin action. Nemenoff *et al.* (42) studied rat epididymal adipocytes and demonstrated with [^{32}P] prelabeled cells, a 30% decreased phosphorylation state of INH-1 with insulin coincident with a 50% decrease in INH-1 bioactivity (42). Conversely, isoproterenol increased the phosphorylation state of INH-1 by 35% coincident with an increase of 25% in INH-1 bioactivity. Foulkes *et al.* (43), using a perfused rat hemicorpus muscle preparation, demonstrated a decreased phosphorylation state of INH-1 with insulin compared to control (28 ± 11 versus 52 ± 12) and an increased phosphorylation state with isoproterenol (73 ± 11) in keeping with the results of Nemenoff *et al.* (42). In a perfused hindlimb rat muscle preparation, Khatra *et al.* (45) were only able to demonstrate the effects of epinephrine to increase INH-1 bioactivity; however, they were not able to detect an insulin effect. In a subsequent report Foulkes *et al.* (46) using the perfused rat muscle hemicorpus preparation reexamined the question of insulin action on INH-1 phosphorylation state carefully controlling the effects of endogenous epinephrine release. They again demonstrated both an action of insulin alone as well as an action of insulin to counteract the effect of submaximal epinephrine to decrease INH-1 phosphorylation state. Further they were able to explain the previous failure of Khatra *et al.* (45) to detect an effect of insulin to dephosphorylate INH-1 as due to differences in perfusion conditions resulting in very low (5-10%) basal phosphorylation state of INH-1 not seen in the other studies (42,43,46). Thus, in three clear published reports, the action of insulin alone and the opposing actions of insulin and of epinephrine to control the phosphorylation state and bioactivity of INH-1 in insulin sensitive tissues were clearly demonstrated. Recent experiments suggest that in fat cells, DARPP-32 is present in 3T3-LI adipocytes and may play an analogous role to INH-1 (47).

In summary, this model system demonstrates that two insulin-regulated inositol glycan putative mediator species act synergistically to control the dephosphorylation of INH-1 and DARPP-32. The resulting cascade model of activation of two phosphatases may help explain the activation with insulin of both phosphatase 1 and 2C reported in the literature (12,13,14) as well as the dephosphorylation with insulin of INH-1 reported by several investigators (42,43,46).

REFERENCES

1. Hizukuri, S., and Larner, J. (1964) *Biochemistry* **3,** 1783–1788.
2. Mackenzie, C. W. III, Bulbulian, G. J., and Bishop, J. S. (1980) *Biochim. et Biophys. Acta* **614,** 413–424.
3. Hiriga, A., Kikuchi, K., Tamura, S., and Tsuiki, S. (1981) *Europ. J. Biochem*. **119,** 503–510.
4. Tamura, S., Lynch, K. R., Larner, J., Fox, J., Yasui, A., Kikuchi, K., Suzuki, Y., and Tsuiki, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1796–1800.
5. Lawson, J. E., Niu, X-D, Browning, K. S., LeTrong, H., Yan, J., and Reed, L. J. (1993) *Biochem* **32,** 8987–8993.
6. Barford, D. *TIBS* **21,** 407–412.
7. Terasawa, T., Kobayashi, T., Murakani, T., Ohnishi, M., Kato, S., Tanaka, O., Kondo, H., Yamamoto, H., Takeuchi, T., and Tamura, S. (1993) *Arch. Biochem. Biophys.* **307.**
8. Das, A. K., Helps, N. R., Cohen, P. T. WP. TP. T. W., and Barford, D. *Embo J.* **15,** 6798–6809.
9. Davies, S. P., Helps, N. R., Cohen, P. T., and Hardie, D. G. (1995) *FEBS Lett* **377,** 421–425.
10. Fukunaga, K., Kobayashi, T., Tamura, S., and Miyamoto, E. (1993) *J. Biochem*. **268,** 133–137.
11. Donella, D. A., Mac Gowan, C. H., Cohen, P., Marchiori, F., Meyer, H. E., and Pinna, L. A. (1990) *Biochim. et Biophys. Acta* **105,** 199–202.
12. Brady, M. J., Bourbonais, F. J., and Saltiel, A. R. (1998). *J. Biol. Chem*. **273,** 14063–14066.
13. Ragolia L., and Begum, N. (1998) *Mol. Cell Biochem*. **182,** 49–58.
14. Ortmeyer, H. K. (1998) *Horm. Metab. Res.* **30,** 1–6.
15. Fonteles, M. C., Huang, L. C., and Larner, J. (1996) *Diabetologia* **39,** 731–734.
16. Y. Pak, Paule, C. R., Bao, Y-D, Huang, L. C., and Larner, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7759–7763.
17. Stoscheck, C. M. (1990) *Enzymol.* **182,** 50–69.
18. Huang, L. C., and Huang, C. (1975) *Biochem*. **14,** 18–24.
19. Huang, L. C. (1974) *Biochem. et Biophys. Acta* **358,** 281–288.
20. Desdouits, F., Siciliano, J. C., Nairn, A. C., Greengard, P, and Gerault, J-A. (1998) *Biochem. J.* **330,** 211–216.
21. Abe, S., Huang, L. C., and Larner, J. (1996) (Patel, M. S.. Roche, T. E., and . Harris, T. E., Eds.), pp. 187–195, Berkhauser, Verlag, Basel.
22. Malchoff, C. D., Huang, L., Gillespie, N., Villar-Palasi, C., Schwartz, C. F. W.., Cheng, K., Hewlett, E., and Larner, J. (1987). *Endocrinol.* **120,** 1327–1337.
23. Ortmeyer, H. K. (1997) *Exp. Clin. Endocrinol. Diabetes* **105,** 19. [Abs.]
24. Lawrence J. C., Jr., and Roach, P. J. (1997) *Diabetes* **46,** 541–547.
25. Walkenbach, R. J., Hazen, R., and Larner, J. (1978) *Mol. Cell. Biochem*. **19,** 31–41.
26. Guinovart, J. J., Lawrence, J. C., Jr., and Larner, J. (1978). *Biochim. et Biophys. Acta* **539,** 181–194.
27. Gabbay, R. A., and Lardy, H. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2218–2222.
28. Gabbay, R. A., and Lardy, H. A. (1984) *J. Biol. Chem*. **259,** 6052–6055.
29. Cohen, P., Alessi, D. R., and Cross, D. A. (1997) *FEBS Lett.* **410,** 3–10.
30. Lawrence, J. C., Jr., and Larner, J. (1978) *J. Biol. Chem*. **253,** 2104–2113.
31. Villar-Palasi, C., and Guinovart, J. J. (1997) *FASEB J.* **11,** 544–558.
32. Larner, J., Galasko, G., Cheng, K., DePaoli-Roach, A. A., Huang, L., Daggy, P., and Kellogg, J. (1979) *Science* **206,** 1408–1410.
33. Larner, J., Huang, L. C., Schwartz, C. F. W., Oswald, A. S., Shen, T. Y., Kinter, M., Tang, G., and Zeller, K. (1988) *Biochem. and Biophys. Res. Comm*. **151,** 1416–1426.
34. Romero, G., Luttrell, L., Rogol, A., Zeller, K., Hewlett, E., and Larner, J. (1998) *Science* **240,** 509–511.
35. Kilgour, E., Larner, J., and Romero, G. (1992) *Biochem. and Biophys. Res. Commun.* **186,** 1151–1157.
36. Kennington, A. S., Hill, C. R., Craig, J., Bogardus, C., Raz, I., Ortmeyer, H. K., Hansen, B. C., Romero, G., and Larner, J. (1990) *New Eng. J. Med.* **323,** 373–378.
37. Shashkin, P. N., Shashkina, E. F., Fenquist-Forbes, F., Zhou, Y. P., Gull, V., and Katz, A. (1997) *Diabetologia* **40,** 557–563.
38. Huang, L. C., Fonteles, M. C., Houston, D. B., Zhang, C., and Larner, J. (1993) *Endocrinol.* **132,** 652–657.
39. Larner, J., and Huang, L. C. (1999) Submitted for publication.
40. Aitken, A., and Cohen, P. (1982) *FEBS Lett.* **147,** 54–58.
41. Hemmings, H. C., Jr., Williams, K. R., Konigsberg, W. H., and Greengard, P. (1984) *J. Biol. Chem*. **259,** 14486–14490.
42. Nemenoff, R. A., Blackshear, P. J., and Avruch, J. (1983) *J. Biol. Chem*. **258,** 9437–9443.
43. Foulkes, J. G., Jefferson, L. S., and Cohen, P. (1980) *FEBS Lett.* **112,** 21–24.
44. Denton, R. M., Midgley, P. J. W., Rutter, G. A., Thomas, A. P., and Mc Cormack, J. G. (1989) *Ann. N.Y. Acad. Sci.* **573,** 85–296.
45. Khatra, B. S., Chiasson, J. L., Shikama, H., Exton, J. H., and Soderling, T. R. (1980) *FEBS Lett.* **114,** 253–256.
46. Foulkes, J. G., Cohen, P., Strada, S. J., Everson, W. V., and Jefferson, L. S. (1982) *J. Biol. Chem*. **257,** 12493–12496.
47. Brady, M. J., Nairn, A. C., and Saltiel, A. R. (1997) *J. Biol. Chem*. **272,** 29698–29703.