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Inositol 1,3,4-Trisphosphate Acts *in Vivo* as a Specific Regulator of Cellular Signaling by Inositol 3,4,5,6-Tetrakisphosphate*

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Ca^{2+}-activated Cl^- channels are inhibited by inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P_4) (Xie, W., Kaetzel, M. A., Bruzik, K. S., Dedman, J. R., Shears, S. B., and Nelson, D. J. (1996) *J. Biol. Chem.* 271, 14092–14097), a novel second messenger that is formed after stimulus-dependent activation of phospholipase C (PLC). In this study, we show that inositol 1,3,4-trisphosphate (Ins(1,3,4)P_3) is the specific signal that ties increased cellular levels of Ins(3,4,5,6)P_4 to changes in PLC activity. We first demonstrated that Ins(1,3,4)P_3 inhibited Ins(3,4,5,6)P_4 1-kinase activity that was either (i) in lysates of AR4–2J pancreatoma cells or (ii) purified 22,500-fold (yield = 13%) from bovine aorta. Next, we incubated [^{3}H]inositol-labeled AR4–2J cells with cell permeant and non-radiolabeled 2,5,6-tri-*O*-butyryl-*myo*-inositol 1,3,4-trisphosphate-hexakis(acetoxymethyl) ester. This treatment increased cellular levels of Ins(1,3,4)P_3 2.7-fold, while [^{3}H]Ins(3,4,5,6)P_4 levels increased 2-fold; there were no changes to levels of other ^{3}H-labeled inositol phosphates. This experiment provides the first direct evidence that levels of Ins(3,4,5,6)P_4 are regulated by Ins(1,3,4)P_3 *in vivo*, independently of Ins(1,3,4)P_3 being metabolized to Ins(3,4,5,6)P_4. In addition, we found that the Ins(1,3,4)P_3 metabolites, namely Ins(1,3)P_2 and Ins(3,4)P_2, were >100-fold weaker inhibitors of the 1-kinase compared with Ins(1,3,4)P_3 itself (IC_{50} = 0.17 μM). This result shows that dephosphorylation of Ins(1,3,4)P_3 *in vivo* is an efficient mechanism to "switch-off" the cellular regulation of Ins(3,4,5,6)P_4 levels that comes from Ins(1,3,4)P_3-mediated inhibition of the 1-kinase. We also found that Ins(1,3,6)P_3 and Ins(1,4,6)P_3 were poor inhibitors of the 1-kinase (IC_{50} = 17 and >30 μM, respectively). The non-physiological trisphosphates, D/L-Ins(1,2,4)P_3, inhibited 1-kinase relatively potently (IC_{50} = 0.7 μM), thereby suggesting a new strategy for the rational design of therapeutically useful kinase inhibitors. Overall, our data provide new information to support the idea that Ins(1,3,4)P_3 acts in an important signaling cascade.

There is considerable interest in the idea that Ins(1,4,5)P_3 [1] and Ins(1,3,4,5)P_4 (Fig. 1) act in a co-ordinated manner as mediators of stimulus-dependent Ca^{2+} mobilization (1, 2). This has naturally led us to consider that the 5-phosphatases that degrade Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (3) are signaling "off-switches." This in turn has created the impression that the pathway by which these two inositol phosphates are dephosphorylated serves only as a conduit that replenishes the free inositol pool. In contrast, we have recently suggested that one of these downstream products, namely Ins(1,3,4)P_3, should be viewed in an important cell-signaling context (4). This new hypothesis comes from the observation that a rat hepatic Ins(3,4,5,6)P_4 1-kinase was inhibited *in vitro* by Ins(1,3,4)P_3 (4, 5). The reason that this effect of Ins(1,3,4)P_3 upon Ins(3,4,5,6)P_4 metabolism is of such interest is that Ins(3,4,5,6)P_4 is an inhibitor of the conductance of the calcium-activated Cl^- channels in the plasma membrane (6–9). These ion channels make important contributions to salt and fluid secretion, and in addition they may participate in osmoregulation, pH balance, and smooth muscle excitability (10–13).

The cellular accumulation of Ins(3,4,5,6)P_4 is known to correlate well with receptor-dependent changes in PLC activity, but the molecular mechanisms that link these two events have not been fully elucidated (14). Our current hypothesis (15, 16) is that cellular levels of Ins(3,4,5,6)P_4 depend upon a dynamic balance between two competing enzyme activities acting in a closed substrate cycle: Ins(1,3,4,5,6)P_5 1-phosphatase and Ins(3,4,5,6)P_4 1-kinase (Fig. 1). The poise of this cycle is proposed to be regulated in such a manner that it can shift in favor of Ins(3,4,5,6)P_4 accumulation whenever PLC is activated, perhaps through inhibition of the Ins(3,4,5,6)P_4 1-kinase by Ins(1,3,4)P_3 (Fig. 1). However, to date such inhibition has only been observed in studies with the purified rat hepatic kinase (4, 5). No direct evidence has previously been published that indicates Ins(1,3,4)P_3 can regulate Ins(3,4,5,6)P_4 1-kinase activity in intact cells; it was a goal of the current study to explore this issue.

In order to investigate if Ins(1,3,4)P_3 can regulate Ins(3,4,5,6)P_4 levels in intact cells, it was necessary to increase cellular levels of Ins(1,3,4)P_3 specifically, under conditions

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[1] The abbreviations used are: InsP_n, inositol polyphosphate, where *n* is the number of phosphates (*e.g.* InsP_3); PEG, polyethylene glycol; PLC, phospholipase C; HPLC, high pressure liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CaM KII, calmodulin-dependent protein kinase; Bt$_3$Ins(1,3,4)P_3/AM, 2,5,6-tri-*O*-butyryl-*myo*-inositol 1,3,4-trisphosphate hexakis (acetoxymethyl) ester.

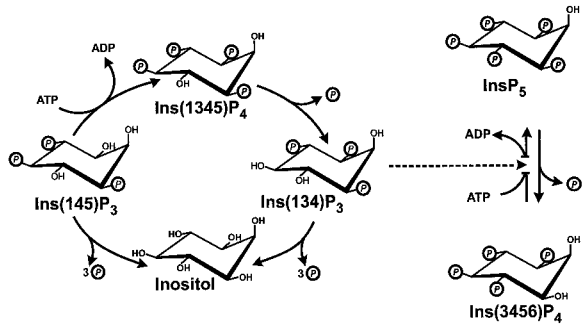

FIG. 1. **Proposed mechanism for the regulation of cellular Ins(3,4,5,6)P_4 levels by Ins(1,3,4)P_3.** The proposed link between Ins(1,3,4)P_3 levels and the interconversion of Ins(1,3,4,5,6)P_5 and Ins(3,4,5,6)P_4 (15, 16) is described. The broken line illustrates the inhibitory effect of Ins(1,3,4)P_3 upon Ins(3,4,5,6)P_4 phosphorylation by the 1-kinase.

where PLC activity was not activated. In this way, we could avoid the possibility of Ins(3,4,5,6)P_4 metabolism also being regulated by the many additional signal transduction processes that are activated downstream of PLC. To this end, the development of cell-permeant, bioactivatable analogues of inositol phosphates (7, 17, 18) has provided us with new opportunities to examine the functions of inositol polyphosphates in intact cells. The charge-masking groups that enable these derivatives to permeate into cells are hydrolyzed by intracellular esterases, releasing the native isomer (7, 17). In this study we used a new cell-permeant analogue, 2,5,6-tri-*O*-butyryl-*myo*-inositol 1,3,4-trisphosphate hexakis(acetoxymethyl) ester (Bt_3Ins(1,3,4)P_3/AM; Ref. 19), to elevate the cellular concentration of Ins(1,3,4)P_3 inside rat pancreatoma (AR4–2J) cells.

This experimental approach was important for another reason. Rather than Ins(1,3,4)P_3 regulating an enzyme of Ins(3,4,5,6)P_4 metabolism (Fig. 1), in principle, Ins(1,3,4)P_3 could instead elevate Ins(3,4,5,6)P_4 concentration simply by being metabolized to it (*i.e.* a mass action effect). For example, Ins(1,3,4)P_3 can be converted to Ins(3,4,5,6)P_4 by the sequential actions of Ins(1,3,4)P_3 6-kinase, Ins(1,3,4,6)P_4 5-kinase, and Ins(1,3,4,5,6)P_5 1-phosphatase (15, 16, 20). Others have proposed an alternative pathway for *de novo* Ins(3,4,5,6)P_4 synthesis, which requires the sequential actions of Ins(1,3,4)P_3 6-kinase, Ins(1,3,4,6)P_4 1-phosphatase, and Ins(3,4,6)P_3 5-kinase (21–23). We have now used AR4–2J cells to examine whether Ins(1,3,4)P_3 alters Ins(3,4,5,6)P_4 levels by mass action effects. Our strategy was based upon first prelabeling the metabolic pool of Ins(3,4,5,6)P_4 to steady-state with [^{3}H]inositol. These cells were then treated with non-radiolabeled Bt_3Ins(1,3,4)P_3/AM. We investigated if there was any significant metabolic flux from Ins(1,3,4)P_3 to Ins(3,4,5,6)P_4, which would have revealed itself by tending to decrease the amount of ^{3}H label in the [^{3}H]Ins(3,4,5,6)P_4 pool, due to a pulse-chase effect (15).

Another feature of an effective signal transduction process relates to its specificity. If the biological effects of a signaling compound cannot be imitated by its products and precursors, this provides sensitivity in the signaling "on" and "off" switches. In the case of signaling by Ins(1,3,4)P_3, the "on-switch" is dephosphorylation of Ins(1,3,4,5)P_4 (3). This process is particularly sensitive, as Ins(1,3,4,5)P_4 is a 290-fold weaker inhibitor of the 1-kinase than is Ins(1,3,4)P_3 (4). We have now turned our attention to considering how effective is the dephosphorylation of Ins(1,3,4)P_3 as a signaling off-switch. *In vivo*, both 4- and 1-phosphatases actively degrade Ins(1,3,4)P_3 to Ins(1,3)P_2 and Ins(3,4)P_2, respectively (24–26). We have therefore determined the potency with which these bisphosphate degradation products inhibit the 1-kinase.

There was one further aspect to this study that is relevant to the development of Ins(3,4,5,6)P_4 agonists and antagonists for pharmacological intervention in the signaling actions of Ins(3,4,5,6)P_4 (18, 27). This goal is directed at diseases that might be treated by either up-regulating or down-regulating Ca^{2+}-activated Cl^- secretion (18, 27). A major challenge to pharmacological intervention at the effector site for Ins(3,4,5,6)P_4 comes from the exquisite specificity with which it blocks Cl^- channel conductance; Ins(1,3,4)P_3, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4, and Ins(1,3,4,5,6)P_5 are all ineffective (6, 8, 9). Moreover, at least one, and possibly both of the OH groups on Ins(3,4,5,6)P_4, are also critical determinants of substrate specificity (18, 28). This tight specificity may make it difficult to rationally design a functional analogue of Ins(3,4,5,6)P_4. A possible alternative therapeutic strategy might be to target Ins(3,4,5,6)P_4 synthesis, rather than its site of action. To this end, we examined the impact on the 1-kinase of some analogues of Ins(1,3,4)P_3 that contain a phosphate group in the 2-position.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Inositol was purchased from American Radiolabeled Chemicals Inc. or NEN Life Science Products. [^{3}H]Ins(1,3,4)P_3 was prepared by dephosphorylating [^{3}H]Ins(1,3,4,5)P_4 (20 Ci/mmol, NEN Life Science Products) with recombinant Ins(1,3,4,5)P_4 5-phosphatase, which was kindly provided by Dr. C. Erneux (29). Ins(1,4)P_2 was purchased from Sigma. D/L-2,5,6-Tri-*O*-butyryl-*myo*-inositol 1,3,4-trisphosphate-hexakis(acetoxymethyl) ester (D/L-Bt_3Ins(1,3,4)P_3/AM) was synthesized as described previously (19). In some experiments, we used enantiomerically pure D-Bt_3Ins(1,3,4)P_3/AM. This compound was prepared from the enantiomerically pure precursor, 4-*O*-benzyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (30). Alkylation of the hydroxy group with benzyl bromide in dimethyl formamide at 50 °C for 20 h and in the presence of an excess of sodium hydride and tetrabutyl ammonium iodide afforded 3,4-di-*O*-benzyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol. Purification by preparative HPLC (92% MeOH, RP-18, 10 μm, 50 × 250 mm, 40 ml/min) gave 55% yield as an oil $[\alpha]_D^{20}$ was +23°; c = 0.75, chloroform). The more labile ketal was removed by a 2-h treatment with acetyl chloride (5%) in a mixture of acetonitrile and methanol (4:5, v/v). The solution was neutralized with triethylamine and evaporated to dryness. The crude material was purified by preparative HPLC (90% MeOH, RP-18, 10 μm, 50 × 250 mm, 40 ml/min) to give 3,4-di-*O*-benzyl-1,2-*O*-cyclohexylidene-*myo*-inositol in 58% yield as a clear oil $[\alpha]_D^{20}$ was −11.1°; c = 0.72, chloroform). The latter was finally converted to D-Bt_3Ins(1,3,4)P_3/AM, as described previously for the racemic precursor (19). Analytical data for D-Bt_3Ins(1,3,4)P_3/AM were as follows: $[\alpha]_D^{20}$ was −7.3° (c = 0.87, toluene). Direct chemical ionization high resolution mass spectroscopy [M-CH_2OAc]$^-$ ($C_{33}H_{52}O_{28}P_3$) gave a calculated m/z of 989.1858; found m/z was 989.1868. ^{1}H and ^{31}P NMR data were in accordance with those of the racemic compound. Enantiomerically pure D-Bt_2Ins(1,4,5,6)P_4/AM was synthesized as described previously (7). All bioactivatable esters were dissolved in Me_2SO/Pluronic (5%, v/v) as described previously (7).

Ins(1,3)P_2, D/L-Ins(3,4)P_2, D/L-Ins(1,2,4)P_3, Ins(1,2,3)P_3, D/L-Ins(1,2,4,6)P_4, and D/L-Ins(1,2,3,4)P_4 were prepared as described previously (31–33). Ins(1,3,6)P_3 and Ins(1,4,6)P_3 were prepared as described previously (34, 35). Sources of other inositol phosphates are given elsewhere (4, 8).

Bombesin, bovine serum albumin, phosphocreatine, phosphocreatine kinase, heparin agarose resin (type II and IIIs), and protease inhibitors were purchased from Sigma. The calmodulin-dependent protein kinase (CaM KII) was obtained from New England Biolabs. Protein kinases A and C, and their assay kits (SpinZyme Format), were the products of Pierce. The UNO Q12 anion exchange column was acquired from Bio-Rad Laboratories. Polyethylene glycol 4000 was purchased from Fluka. Frozen bovine aorta were purchased from Pel-Freez Biological.

Assay of Ins(3,4,5,6)P_4 1-Kinase—The 1-kinase activity was assayed as described before (4). Briefly, 10–20 μl of enzyme was incubated at 37 °C in a final volume of 100 μl containing about 4000 dpm [^{3}H]Ins(3,4,5,6)P_4, which was adjusted to a concentration of 0.25 μM with non-radioactive substrate, 20 mM HEPES (pH 7.2), 6 mM $MgSO_4$, 0.4 mg/ml saponin, 100 mM KCl, 0.3 mg/ml bovine serum albumin, 2 μM $InsP_6$, 5 mM ATP, 10 mM phosphocreatine, and 2.5 Sigma units of phosphocreatine kinase. After 30 min., the reaction was stopped by quenching with 1 ml of ice-cold medium containing 1 mg/ml $InsP_6$, 0.2

M ammonium formate, and 0.1 M formic acid. The quenched reactions were diluted to 10 ml with deionized water, and chromatographed on Bio-Rad gravity-fed columns using AG 1-X8 ion exchange resin.

For some assays, the 1-kinase was preincubated at 30 °C for 10 min with (*a*) 125 units of the catalytic subunit of protein kinase A, (*b*) 0.2 unit of protein kinase C, (*c*) 600 units of calmodulin, or (*d*) 500 units of CaM KII, preactivated with calmodulin/Ca^{2+} (New England Biolabs). The protein kinases used in these experiments were all shown to be active in control experiments (assay kits for protein kinases A and C were supplied by Pierce; the CaM KII was checked using a kit purchased from Upstate Biochemicals).

The 1-kinase was also used as a diagnostic tool to verify the nature of HPLC-purified [^{3}H]Ins(3,4,5,6)P_4. In these incubations, 45 μl of purified 1-kinase was added to 225 μl of medium containing 67 mM HEPES (pH 8.0 with KOH), 0.7 mM EDTA, 8.7 mM $MgSO_4$, 6.7 mM ATP, 13.3 mM phosphocreatine, 1.33 μM $InsP_6$, and 6 Sigma units of phosphocreatine kinase. Then, 30 μl of the appropriate HPLC fraction was added (which brought the final pH to approximately 6.5). Reactions (at 37 °C) were allowed to proceed to completion (over a 3-h period), and then the amount of [^{3}H]$InsP_5$ formed was determined using gravity-fed ion-exchange columns, as described above.

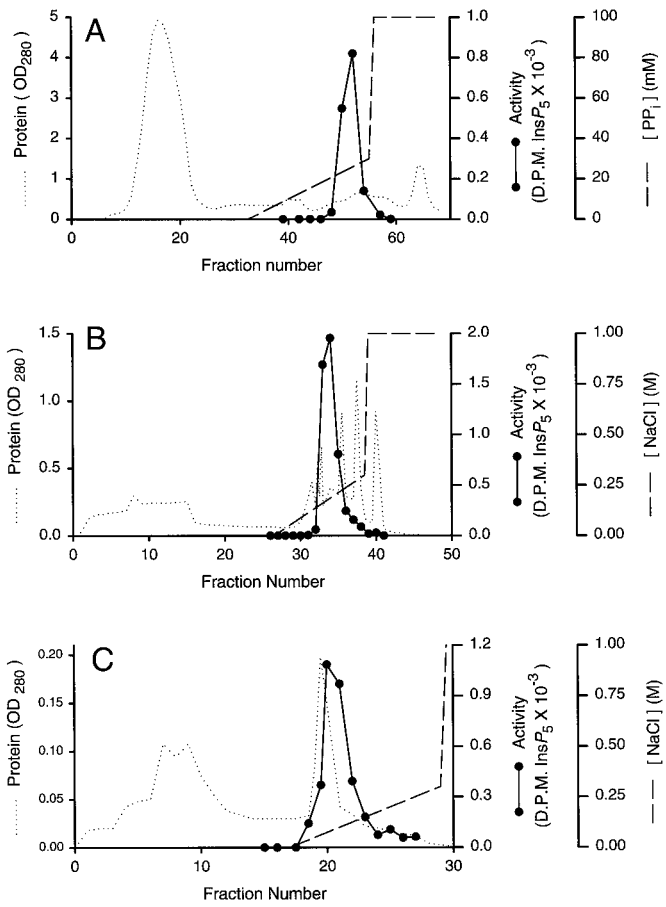

FIG. 2. **Purification of Ins(3,4,5,6)P_4 1-kinase.** The 1-kinase was purified as described under "Experimental Procedures" by subjecting a 10–30% PEG precipitate to Heparin II-agarose affinity chromatography (*panel A*), followed by UNO Q12 anion exchange chromatography (*panel B*) and Heparin IIIS affinity chromatography (*panel C*). Data are representative of three experiments.

Purification of Ins(3,4,5,6)P_4 1-Kinase—Frozen bovine aortas were thawed on ice, the attached fat was removed, and then the aorta were pulverized in a meat grinder. In a typical preparation, 300–350 g of ground aortas were homogenized in two volumes of 50 mM bis-Tris (pH 7.0), 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride in a tissue blender.

The homogenate was filtered through four layers of cheesecloth, and a 10–30% (w/v) polyethylene glycol 4000 precipitate was prepared. The resultant pellet was resuspended in 100 ml of Buffer A containing 50 mM bis-Tris (pH 7.0), 1 mM EGTA, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The suspension was filtered and loaded at a flow rate of 1 ml/min onto a heparin-agarose type II column (3.2 × 24 cm). After washing with 300 ml of Buffer A at flow rate of 1.5 ml/min, the bound protein was eluted with a linear gradient of 0–30 mM of sodium pyrophosphate in Buffer A.

The peak fractions of enzyme activity eluted from the heparin column were pooled, then frozen and stored at −70 °C. Either two or three preparations were subsequently thawed and combined, dialyzed against 2 liters of 25 mM bis-Tris (pH 7.0) at 4 °C for 3 h, and loaded onto a UNO Q12 anion exchange column (1.5 × 6.8 cm), which was pre-equilibrated with 100 ml of Buffer A. A constant flow rate of 0.5 ml/min was maintained throughout the chromatography. After washing with 60 ml of Buffer A, the bound protein was eluted with a linear gradient of Buffer A plus 0–300 mM NaCl, followed by 60 ml of Buffer A plus1 M NaCl.

Peak fractions of enzyme activity eluted from the UNO Q12 column were pooled, dialyzed against 2 liters of 25 mM bis-Tris (pH 7.0) at 4 °C for 3 h, and loaded on to heparin-agarose type IIIs (1.1 × 13.5 cm), which was pre-equilibrated with 50 ml of Buffer A. A constant flow rate of 0.5 ml/min was maintained throughout. After washing with 60 ml of Buffer A, the bound protein was eluted with a linear gradient of 0–300 mM NaCl in Buffer A, followed by 60 ml of 1 M NaCl in Buffer A.

The protein concentration of the 1-kinase preparation was determined using Bio-Rad Protein Assay Dye Reagent with bovine serum albumin as standard. Final enzyme preparations were stored in 10% glycerol plus 1 mg/ml bovine serum albumin at −70 °C.

Gel Filtration—A 1-ml aliquot of a resuspension of a 10–30% PEG precipitation was loaded at a flow rate of 0.25 ml/min to Sephacryl S100 column (2.0 × 86 cm), which was pre-equilibrated with 600 ml of bis-Tris buffer containing 50 mM bis-Tris (pH 7.0), 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 mM NaCl. The protein was then chromatographed using the same buffer at a constant 0.25 ml/min flow rate. Fractions (5ml) were collected and assayed for enzyme activity. The column was calibrated under the exactly same conditions using bovine serum albumin, chicken ovalbumin, equine myoglobin, and vitamin B-12.

Culturing and Incubation of AR4–2J Cells—The AR4–2J pancreatoma cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, 10% fetal bovine serum, 2 mM glutamine, 500 units/ml penicillin, and 500 μg/ml streptomycin, with 10% conditioned medium, and harvested by brief trypsinization. Either 2.0 × 10^5 or 1.2 × 10^6 cells were seeded in 24-well or 6-well tissue culture plates, respectively. Cells were labeled with 75–150 μCi/ml [^{3}H]*myo*-inositol for 4 days (medium was replaced on the 3rd day) in 700 μl (for 24-well plates) or 3 ml (for 6-well plates) of the above culture medium. After completion of the labeling protocol, the culture medium was aspirated and the cells were washed twice with Krebs/Ringer/HEPES solution (15). Cells were then incubated in 300 μl (for 24-well) or 1 ml (for 6-well) of Krebs/Ringer/HEPES solution for 2 h. Then 20 mM LiCl was added, and 20 min later cells were treated for the indicated time with (i) a cell-permeant inositol phosphate, (ii) vehicle, or (iii) bombesin.

Cells were quenched and neutralized, and the inositol phosphates were separated by HPLC as described elsewhere (36). Radioactivity was either counted on-line, using a Radiomatic Flo-1, or recovered in 1-ml

TABLE I
Purification of Ins(3,4,5,6)P_4 1-kinase

See under "Experimental Procedures" for details of the purification procedures.

Procedure	Protein	Volume	Specific activity	Purification	Apparent yield
	mg	*ml*	*pmol/ng/min*	*-fold*	*%*
Homogenate	1949	389	0.3	1	100
10–30% PEG	282	100	2.32	7.7	113
Heparin II	11	60	28.5	95	55
UNO	0.69	15	137	457	16
Heparin IIIS	0.011	10	6755	22,516	13

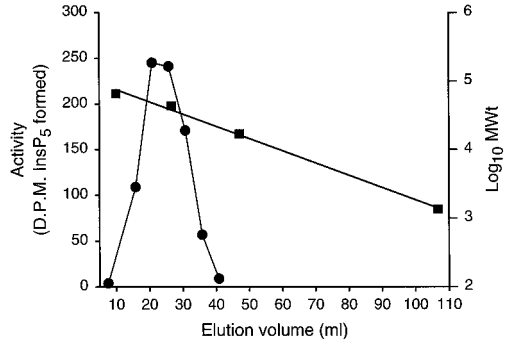

FIG. 3. **Molecular size of the Ins(3,4,5,6)P_4 1-kinase.** The 1-kinase was analyzed by size-exclusion chromatography as described under "Experimental Procedures". Enzyme activity is indicated by the *circles*; the *squares* depict the elution positions of standards: serum albumin albumin (66 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa).

fractions. The levels of ^{3}H-labeled inositol phosphates were normalized as a ratio to cellular levels of [^{3}H]InsP_6; the latter were unaffected by any of the experimental protocols performed in this study.

For some experiments, after the AR4-2J cells were harvested, cells were collected by centrifugation in serum-containing culture medium. The pellet was washed in HEPES-buffered saline, and then a lysate was prepared by resuspending the packed cells in an equal volume of ice-cold lysis buffer comprising: 50 mM KCl, 50 mM HEPES (pH 7.2), 1 mM EDTA, 5 mM ATP, 4 mM CHAPS, 0.4 mM phenylmethylsulfonyl fluoride, 40 μM E-64, 10 μM leupeptin, 3 μM pepstatin.

Assay of Mass Levels of Ins(1,3,4)P_3 inside AR4–2J Cells—Following the HPLC fractionation of extracts of [^{3}H]inositol-labeled cells (see above), 1-ml fractions were saved, from which 25-μl aliquots were counted for radioactivity so as to identify the Ins(1,3,4)P_3 peak (because of the low levels of endogenous [^{3}H]Ins(1,3,4)P_3, samples were "spiked" with 4000 dpm [^{3}H]Ins(1,3,4)P_3 (20 Ci/mmol) before they were applied to the HPLC column). The Ins(1,3,4)P_3 peak was then desalted (37) and resuspended in 60 μl Ins(1,3,4)P_3 6-kinase assay buffer: 50 mM KCl, 50 mM HEPES, pH 7.2, 10 mM phosphocreatine, 6 mM ATP, 8 mM $MgSO_4$, 25 Sigma units/ml phosphocreatine kinase, 0.5 mg/ml bovine serum albumin. Recovery of [^{3}H]Ins(1,3,4)P_3 from the cell extract was typically 70–75%. Each Ins(1,3,4)P_3 sample was then divided into two equal portions, named A and B. The Ins(1,3,4)P_3 was depleted from portion B by its incubation for 60 min at 37 °C with 0.1 μg of recombinant Ins(1,3,4)P_3 1-phosphatase (kindly supplied by Dr. J. York, Duke University, Durham, NC). Control experiments showed that the extent of Ins(1,3,4)P_3 hydrolysis exceeded 95%. The Ins(1,3,4)P_3 1-phosphatase was then heat-inactivated (3 min at 100 °C). Aliquots (9 μl) of either portion A or B were then incubated in triplicate for 20 min in a total volume of 50 μl containing purified Ins(1,3,4)P_3 6-kinase, its assay buffer (see above), plus approximately 4000 dpm [^{3}H]Ins(1,3,4)P_3. Other incubations (in quadruplicate) were performed that, in place of the Ins(1,3,4)P_3 from a cell extract, contained between 0.1 and 50 pmol of non-radiolabeled Ins(1,3,4)P_3. A standard curve was constructed from the decrease in the phosphorylation of [^{3}H]Ins(1,3,4)P_3 to [^{3}H]Ins(1,3,4,6)P_4 that was observed as the Ins(1,3,4)P_3 mass was increased. The difference in Ins(1,3,4)P_3 mass values between portions A and B (*i.e.* the amounts hydrolyzed by the Ins(1,3,4)P_3 1-phosphatase) were taken to represent the quantity of Ins(1,3,4)P_3 in the original HPLC-purified sample.

RESULTS

Purification and Properties of the Ins(3,4,5,6)P_4 1-Kinase from Bovine Aorta—In our earlier study with the Ins(3,4,5,6)P_4 1-kinase in rat liver, the enzyme was found to be extremely labile, and we were only able to elicit a 1600-fold purification with a 1% yield (4). No other laboratory has published a purification protocol for this enzyme. We developed a new strategy for the current study, the most notable aspect of which was the efficiency of an affinity purification step using heparin IIIS (Fig. 2; Table I). Thus, using homogenates of bovine aortas as starting material, we purified the 1-kinase 22,500-fold with a 13% yield (Fig. 2; Table I). Our preparations of 1-kinase have an affinity for Ins(3,4,5,6)P_4 (0.1–0.2 μM, data not shown) that

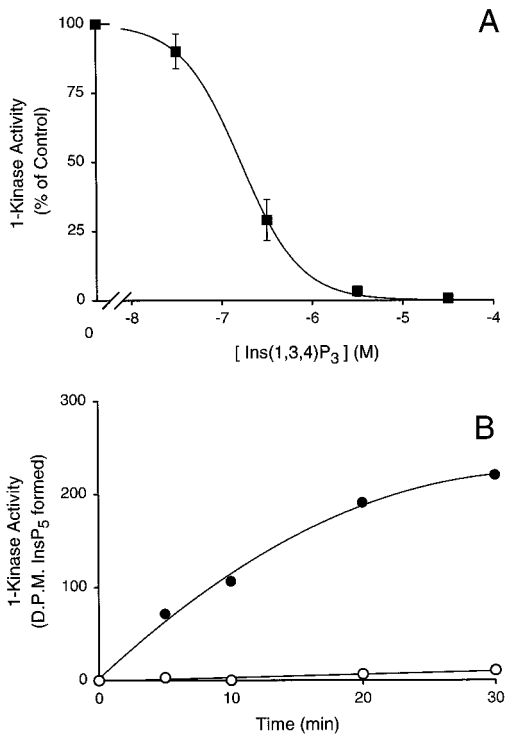

FIG. 4. **Inhibition, by Ins(1,3,4)P_3, of the Ins(3,4,5,6)P_4 1-kinase from bovine aorta and AR4–2J pancreatoma cells.** *Panel A*, Ins(3,4,5,6)P_4 1-kinase activity was assayed as described under "Experimental Procedures," using either the enzyme purified from bovine aorta incubated with the indicated concentrations of Ins(1,3,4)P_3 (n = 3, *vertical bars* denote standard errors) (*panel A*), or lysates of AR4–2J cells in medium containing either 10 μM Ins(1,3,4)P_3 (*open circles*) or no Ins(1,3,4)P_3 (*closed circles*) (*panel B*); a single experiment is shown, representative of two.

is very similar to the substrate affinity of the rat hepatic enzyme (4). Gel filtration indicated the size of the enzyme to be 46 kDa (Fig. 3), which is also similar to that of the rat liver enzyme (4). The 1-kinase was strongly inhibited by Ins(1,3,4)P_3 (IC_{50} = 0.17 μM, Fig. 4*A*). The enantiomer of Ins(1,3,4)P_3, namely Ins(1,3,6)P_3, was a 100-fold weaker inhibitor of the Ins(3,4,5,6)P_4 1-kinase (Table II). The activity of the purified Ins(3,4,5,6)P_4 1-kinase was unaffected when 20 mM KCl in the incubation buffer was substituted with 20 mM LiCl (data not shown).

The purified 1-kinase was reconstituted with either protein kinase A, protein kinase C, Ca^{2+}/calmodulin, or CaM KII. In no case was there any modification to 1-kinase activity, nor was there any effect upon the potency of inhibition by Ins(1,3,4)P_3 (data not shown). Positive controls for each of these protein kinases were obtained by verifying their activities using appropriate assay kits (see "Experimental Procedures").

The Effect of Bt$_2$Ins(1,3,4)P_3/AM upon [^{3}H]Inositol Phosphates in AR4–2J Cells—One aim of this study was to use intact cells to examine the physiological relevance of the inhibition of the Ins(3,4,5,6)P_4 1-kinase by Ins(1,3,4)P_3, since this effect has only previously been observed with the isolated enzyme (4, 5). For these experiments we used a cell-permeant and bioactivatable analogue of Ins(1,3,4)P_3. There are several important aspects of the experimental protocol that should be emphasized. First, we chose to perform these studies with AR4–2J pancreatoma cells. This decision reflects the fact that, in these cells, Ins(1,4,5)P_3 is primarily metabolized by the 5-phosphatase pathway; the Ins(1,4,5)P_3 3-kinase is a quantitatively minor metabolic route (38, 39), so the ensuing downstream products, including Ins(1,3,4)P_3, are present at unusually low levels in "resting" cells (15). This low base line

TABLE II
Relative potencies of inositol phosphates as inhibitors of the Ins(3,4,5,6)P_4 1-kinase

The potencies with which various inositol phosphates inhibited the Ins(3,4,5,6)P_4 1-kinase were determined as described under "Experimental Procedures." Data are presented as IC_{50} values (means and standard errors, with numbers of experiments in parentheses).

Inositol phosphate	IC_{50}
	μM
Ins(1,3,4)P_3	0.17 ± 0.05 (3)
D/L-Ins(1,2,4)P_3	0.7 ± 2 (4)
D/L-Ins(1,2,3,4)P_4	1.6 ± 0.6 (4)
Ins(1,2,3)P_3	4.2 ± 0.9 (3)
Ins(1,3,6)P_3	17 ± 7 (3)
D/L-Ins(1,2,4,6)P_4	16.7 ± 4.7 (4)
Ins(1,4,5,6)P_4	>30 (3)
Ins(1,4,6)P_3	>30 (3)

improves the "signal-to-noise" ratio, making our experiments more sensitive to small experimental manipulations of Ins(1,3,4)P_3 levels. We also checked that the Ins(3,4,5,6)P_4 1-kinase in these cells was sensitive to inhibition by Ins(1,3,4)P_3. In cell lysates, 10 μM Ins(1,3,4)P_3 inhibited the rate of phosphorylation of Ins(3,4,5,6)P_4 by greater than 95% (Fig. 4*B*).

A second aspect of our experimental protocol that is worth emphasizing is that we preincubated cells with [^{3}H]inositol for 4 days. At this point the cellular pool of Ins(3,4,5,6)P_4 was radiolabeled to steady state (16, 39). Thus, any increases in [^{3}H]Ins(3,4,5,6)P_4 that we observed truly reflect elevated mass levels of this polyphosphate. Third, we were concerned that cell-permeant analogues of inositol phosphates are typically de-esterified relatively slowly (17), such that a rapid rate of Ins(1,3,4)P_3 dephosphorylation would act to prevent the accumulation of this compound inside cells. Our cell incubation medium was therefore supplemented with lithium, so as to inhibit the Ins(1,3,4)P_3 1-phosphatase (40). However, it should be noted that this is only a partial solution of this particular problem, since lithium does not inhibit the less active, alternative pathway of Ins(1,3,4)P_3 dephosphorylation by a 4-phosphatase (41). Control experiments indicated that this lithium treatment did not affect levels of [^{3}H]Ins(3,4,5,6)P_4 (data not shown).

Extracts of [^{3}H]inositol-labeled control cells were resolved by HPLC, and the various ^{3}H-labeled inositol phosphates were assayed using an on-line scintillation counter (Fig. 5, *upper panel*). We also analyzed extracts from cells treated with 200 μM D/L-Bt$_3$Ins(1,3,4)P_3/AM (Fig. 5, *lower panel*). In these experiments, any metabolic conversion of Ins(1,3,4)P_3 to Ins(3,4,5,6)P_4 would, by a pulse-chase effect, tend to decrease the amount of [^{3}H]label in the Ins(3,4,5,6)P_4 pool (15). In fact, the opposite result was obtained; the size of the [^{3}H]Ins(3,4,5,6)P_4 peak increased about 2-fold (Fig. 5, Table III). The elevation in [^{3}H]Ins(3,4,5,6)P_4 levels in our experiments (Fig. 5) cannot be caused by an increased flux of non-radiolabeled Ins(1,3,4)P_3 into the [^{3}H]Ins(3,4,5,6)P_4 metabolic pool. Note also that there were no significant changes in the sizes of other [^{3}H]inositol-labeled peaks after treatment with D/L-Bt$_3$Ins(1,3,4)P_3/AM. To control for the possibility of nonspecific effects of cell-permeant inositol phosphates, we also incubated AR4–2J cells with 200 μM D-Bt$_2$Ins(1,4,5,6)P_4/AM (which does not affect 1-kinase activity, see below), and this had no effect upon the [^{3}H]inositol-polyphosphate profiles (data not shown).

We were only able to synthesize limited amounts of Bt$_3$Ins(1,3,4)P_3/AM, and so we did not have sufficient material to perform detailed dose-response curves or time courses. However, we did observe that the treatment of cells with 400 μM D/L-Bt$_3$Ins(1,3,4)P_3/AM approximately doubled the elevation in

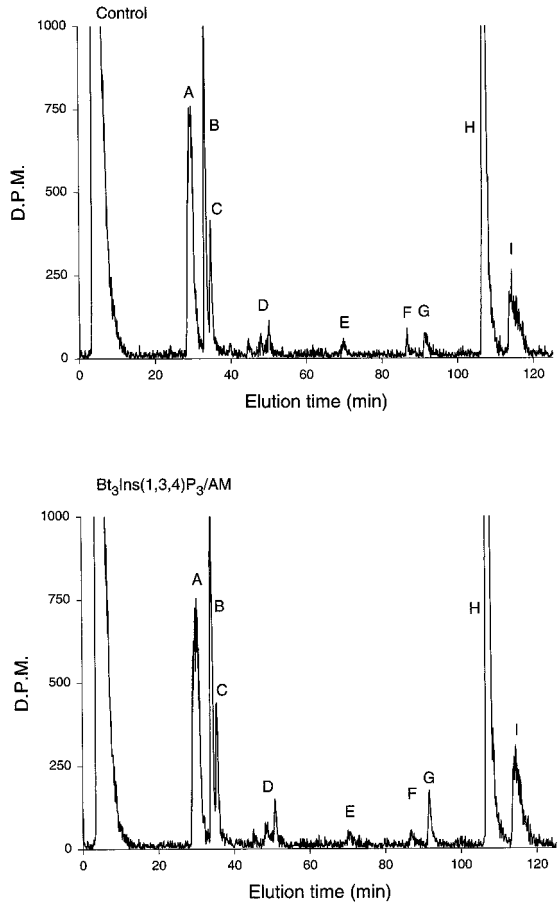

FIG. 5. **The effect of D/L-Bt$_3$ Ins(1,3,4)P_3/AM upon ^{3}H-labeled inositol phosphates in AR4–2J cells.** [^{3}H]Inositol-labeled AR4–2J cells were incubated with D/L-Bt$_3$Ins(1,3,4)P_3/AM or vehicle for 60 min, and then the cells were quenched and the inositol phosphates resolved by HPLC, as described under "Experimental Procedures." The data are from one of four representative experiments. The identities of the various peaks (see Ref. 36) are as follows: *A*, GroPIns; *B*, Ins1P; *C*, Ins4P; *D*, InsP_2; *E*, Ins(1,4,5)P_3; *F*, Ins(1,3,4,6)P_4; *G*, Ins(3,4,5,6)P_4; *H*, InsP_5; *I*, InsP_6. The integrated value of the Ins(3,4,5,6)P_4 peaks were 640 dpm (*upper panel*, vehicle) and 1505 dpm (*lower panel*, D/L-Bt$_3$Ins(1,3,4)P_3/AM-treated).

[^{3}H]Ins(3,4,5,6)P_4 levels, compared with the effect of 200 μM D/L-Bt$_3$Ins(1,3,4)P_3/AM; 20 μM D/L-Bt$_3$Ins(1,3,4)P_3/AM did not affect [^{3}H]Ins(3,4,5,6)P_4 levels (data not shown). One factor that must be taken into account when using these types of cell-permeant analogues is the relatively slow rate of their activation by intracellular esterases (17).

The Ins(3,4,5,6)P_4 1-kinase was used as a diagnostic tool so as to confirm the identity of the [^{3}H]Ins(3,4,5,6)P_4 peak that eluted from the HPLC. For these experiments, we did not assay [^{3}H]inositol phosphates by on-line scintillation counting. Instead, individual HPLC fractions were collected. The amount of material loaded onto the HPLC column was increased by culturing greater numbers of cells in larger wells (see "Experimental Procedures"). Thus, the quantity of [^{3}H]Ins(3,4,5,6)P_4 was larger than in the experiments described by Fig. 5. Aliquots of the putative [^{3}H]Ins(3,4,5,6)P_4 peak were incubated with the purified 1-kinase (see "Experimental Procedures"). The [^{3}H]InsP_5 formed, after the assays had been allowed to proceed to completion, was used to identify the amounts of [^{3}H]Ins(3,4,5,6)P_4 in the original cell extracts: 6964 ± 35 dpm from control cells, and 13,322 ± 131 dpm after treatment with D/L-Bt$_3$Ins(1,3,4)P_3/AM. These experiments confirm that levels of [^{3}H]Ins(3,4,5,6)P_4 in intact cells were elevated 2-fold by Ins(1,3,4)P_3.

TABLE III

The effect of D/L-Bt_3Ins(1,3,4)P_3/AM upon levels of ^{3}H-labeled inositol phosphates in AR4–2J cells

Data are collated from four experiments with control and D/L-Bt_3Ins(1,3,4)P_3/AM-treated cells, performed as described by the legend to Fig. 5. Data are expressed as a ratio to [^{3}H]InsP_6 (see "Experimental Procedures"). * indicates the effect of D/L-Bt_3Ins(1,3,4)P_3/AM upon [^{3}H]Ins(3,4,5,6)P_4 levels was statistically significant (p = 0.003).

Inositol phosphate	Control	D/L-Bt_3Ins(1,3,4)P_3/AM
Ins(1,4,5)P_3	0.049 ± 0.008	0.053 ± 0.01
Ins(1,3,4,6)P_4	0.049 ± 0.008	0.048 ± 0.01
Ins(3,4,5,6)P_4	0.096 ± 0.007	0.19 ± 0.006*
InsP_5	4.7 ± 0.49	4.5 ± 0.38

We next investigated if the treatment of AR4–2J cells with Bt_3Ins(1,3,4)P_3/AM elicited increases in levels of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 that were physiologically relevant. For these experiments, we compared the effects of the cell-permeant analogue with those of receptor-dependent activation of PLC, using bombesin as the agonist. [^{3}H]Inositol-labeled AR4–2J cells were treated for 20 min with 200 nM bombesin, or for 60 min with either 100 μM D-Bt_3Ins(1,3,4)P_3/AM, or vehicle. The cell-permeant derivative used in these experiments was from a batch that was different from that used in the experiments described above. This particular batch of the analogue was also enantiomerically pure, and therefore it was used at half the concentration of the D/L-Bt_3Ins(1,3,4)P_3/AM used in the experiments described above. In three experiments, 60-min treatment of AR4–2J cells with 100 μM D-Bt_3Ins(1,3,4)P_3/AM elevated [^{3}H]Ins(3,4,5,6)P_4 levels 1.9 ± 0.3-fold (Fig. 6), which is not significantly different from the results obtained with 200 μM D/L-Bt_3Ins(1,3,4)P_3/AM (Table III). The fact that these changes in Ins(3,4,5,6)P_4 levels were within a physiologically relevant range was confirmed by comparison with the effects of bombesin, which led to a nearly 5-fold increase in [^{3}H]Ins(3,4,5,6)P_4 levels (Fig. 6).

We also compared the effects of D-Bt_3Ins(1,3,4)P_3/AM and bombesin upon cellular Ins(1,3,4)P_3 levels (see under "Experimental Procedures"). Stimulation of AR4–2J cells with bombesin elicited a 13-fold increase in levels of Ins(1,3,4)P_3 (Fig. 6); treatment with D-Bt_3Ins(1,3,4)P_3/AM elevated Ins(1,3,4)P_3 levels 2.7-fold, an effect that may therefore be considered to be within the physiologically relevant range (Fig. 6).

Effects of InsP_2 isomers on Ins(3,4,5,6)P_4 1-Kinase Activity—There is another important issue that is relevant to our evaluation of the significance of Ins(1,3,4)P_3 as an intracellular signal. The extent to which metabolites of Ins(1,3,4)P_3 also inhibit the 1-kinase contributes to the efficiency with which the cell "switches off" the Ins(1,3,4)P_3 signal. The major route of Ins(1,3,4)P_3 metabolism *in vivo* is by its dephosphorylation, by separate 4- and 1-phosphatases, to Ins(1,3)P_2 and Ins(3,4)P_2, respectively (24–26). We therefore determined the potency with which these bisphosphate degradation products inhibited the 1-kinase, relative to Ins(1,3,4)P_3. The IC_{50} for Ins(1,3,4)P_3 was 0.17 μM (Table II and Fig. 4*A*). In contrast, both Ins(1,3)P_2 and D/L-Ins(3,4)P_2 did not significantly affect 1-kinase activity until their concentration exceeded 3 μM (Fig. 7*A*); therefore, these bisphosphates were at least 100-fold weaker inhibitors. Thus, dephosphorylation of Ins(1,3,4)P_3 is a very effective off-switch for relieving inhibition of 1-kinase activity.

The product of 5-phosphatase attack upon Ins(1,4,5)P_3, namely Ins(1,4)P_2, was found to be a poor inhibitor of the 1-kinase (Fig. 7*A*). This is also an important observation that demonstrates that it specifically requires Ins(1,4,5)P_3 metabolism through the 3-kinase pathway to yield an inhibitor of the Ins(3,4,5,6)P_4 1-kinase. In addition, this result shows that all three phosphates of Ins(1,3,4)P_3 contribute substantially to the specificity of inhibition of the 1-kinase.

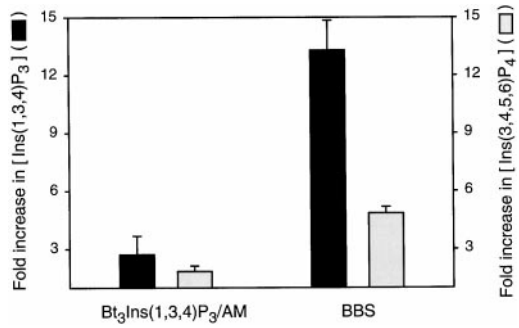

FIG. 6. **Effect of bombesin and D-Bt_3 Ins(1,3,4)P_3/AM upon levels of [^{3}H]Ins(3,4,5,6)P_4 and non-radiolabeled Ins(1,3,4)P_3 in intact AR4–2J cells.** [^{3}H]Inositol-labeled AR4–2J cells were incubated with either (i) 200 nM bombesin (*BBS*) or vehicle for 20 min, or (ii) 100 μM D-Bt_3Ins(1,3,4)P_3/AM or vehicle for 60 min. The cells were quenched and the inositol phosphates resolved by HPLC, as described under "Experimental Procedures." The changes in levels of [^{3}H]Ins(3,4,5,6)P_4 (*gray bars*) and non-radiolabeled Ins(1,3,4)P_3 (*black bars*) were then determined as described under "Experimental Procedures." Means and standard errors are from three experiments.

The Contribution of the 2-Phosphate Group to Inhibition of the 1-Kinase—We found that, in an appropriate context, the addition of a 2-phosphate to the inositol ring could make a positive contribution to inhibitory potency; this is illustrated by the observation that Ins(1,2,3)P_3 was a more potent inhibitor of the 1-kinase (IC_{50} = 4.2 μM, Table II) than was Ins(1,3)P_2 (IC_{50} > 30 μM, Fig. 7*A*). D/L-Ins(1,2,3,4)P_4 was also a relatively potent 1-kinase inhibitor (IC_{50} = 1.6 μM, Table II). D- and/or L-Ins(1,2,3,4)P_4 and Ins(1,2,3)P_3 are found in mammalian cells, the latter at concentrations of up to 10 μM (15, 42, 43). These polyphosphates may be formed by dephosphorylation of InsP_6 (15, 42, 43). Among the inositol phosphates that we tested that contain a 2-phosphate (Table II), D/L-Ins(1,2,4)P_3 was the most potent inhibitor of the 1-kinase (IC_{50} = 0.7 μM, Fig. 7*B*). Neither D- nor L-Ins(1,2,4)P_3 have been detected in mammalian cells, even under circumstances where, had they been present, they should have revealed themselves to some detailed structural analyses (43). As the inhibitory action of Ins(1,3,4)P_3 upon the 1-kinase was so effectively imitated by D/L-Ins(1,2,4)P_3 (Table II), the latter could be a useful new starting point for developing drugs that might intervene in the 1-kinase/1-phosphatase cycle with therapeutic benefit.

DISCUSSION

Two aspects of this study are of particular importance to improving our understanding of the role of Ins(1,3,4)P_3 as an intracellular signal. First, by using a new, cell-permeant, bioactivatable form of Ins(1,3,4)P_3, we have shown that Ins(1,3,4)P_3 elevates levels of Ins(3,4,5,6)P_4 inside intact cells, without Ins(1,3,4)P_3 being metabolized to Ins(3,4,5,6)P_4. Second, we have discovered that Ins(1,3,4)P_3-mediated inhibition of the Ins(3,4,5,6)P_4 1-kinase is very efficiently switched off by dephosphorylation of Ins(1,3,4)P_3. Prior to this study, there had not been a consensus of opinion concerning the mechanism by which PLC activation is coupled to increases in Ins(3,4,5,6)P_4 levels. This is an important issue to resolve, if we are to understand how the cell regulates Ins(3,4,5,6)P_4-mediated inhibition of conductance through Ca^{2+}-activated Cl^- channels (6, 8). We (15) have previously suggested that Ins(3,4,5,6)P_4 levels are controlled by PLC-initiated regulation of the activities of a Ins(1,3,4,5,6)P_5 1-phosphatase/Ins(3,4,5,6)P_4 1-kinase substrate cycle (Fig. 1). Others have argued that Ins(1,3,4)P_3 is converted to Ins(3,4,5,6)P_4 by the sequential actions of Ins(1,3,4)P_3 6-kinase, Ins(1,3,4,6)P_4 1-phosphatase, and Ins(3,4,6)P_3 5-kinase (22, 23, 44). An in-

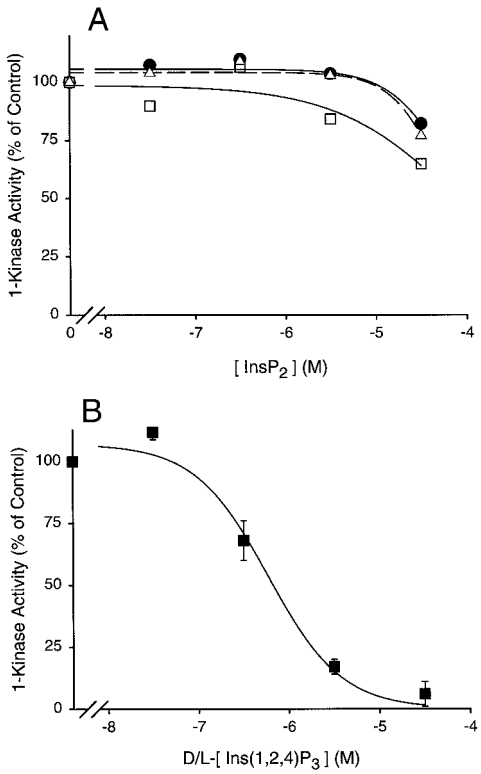

FIG. 7. **Effects of $InsP_2$ isomers and D/L-Ins(1,2,4)P_3 upon Ins(3,4,5,6)P_4 1-kinase activity.** 1-Kinase activity was assayed as described under "Experimental Procedures," in the presence of the following non-radiolabeled inositol phosphates: D/L-Ins(3,4)P_2 (*panel A*, *open squares*), Ins(1,3)P_2 (*panel A*, *triangles*), Ins(1,4)P_2 (*panel A*, *circles*), D/L-Ins(1,2,4)P_3 (*panel B*). Enzyme activity in each case is presented as a percentage of activity in the absence of any inhibitor. Data are derived from three or four experiments, and *vertical bars* denote standard errors (omitted from the experiments with $InsP_2$ isomers for the sake of clarity).

creased metabolic flux from Ins(1,3,4)P_3 to Ins(3,4,5,6)P_4 could elevate Ins(3,4,5,6)P_4 levels by mass action effects. D/L-Bt_3Ins(1,3,4)P_3/AM enabled us to test this idea.

D/L-Bt_3Ins(1,3,4)P_3/AM provided a means of delivering a pulse of non-radiolabeled Ins(1,3,4)P_3 into intact [^{3}H]inositol-labeled cells. If, as a consequence of this treatment, there had been a significant metabolic flux from Ins(1,3,4)P_3 into the Ins(3,4,5,6)P_4 pool, this would have been expected to *decrease* the [^{3}H]label in this Ins(3,4,5,6)P_4 pool by a pulse-chase effect (15). In fact, the opposite result was obtained; D/L-Bt_3Ins-(1,3,4)P_3/AM *elevated* intracellular levels of [^{3}H]Ins(3,4,5,6)P_4 (Table III). Thus, for the first time, we have shown that Ins(1,3,4)P_3 regulates levels of Ins(3,4,5,6)P_4 inside cells, without Ins(1,3,4)P_3 being metabolized to Ins(3,4,5,6)P_4.

An Ins(1,3,4)P_3-mediated inhibition of Ins(3,4,5,6)P_4 1-kinase in intact cells would be expected to alter the poise of the substrate cycle that interconverts Ins(3,4,5,6)P_4 with Ins(1,3,4,5,6)P_5 (Fig. 1). There was not a statistically significant decrease in the levels of [^{3}H]Ins(1,3,4,5,6)P_5 after treatment with D/L-Bt_3Ins(1,3,4)P_3/AM (Table III), but this is not unexpected. The [^{3}H]Ins(1,3,4,5,6)P_5 pool is relatively large, and it is difficult to detect the small changes in its size that are sufficient to support a 2-fold increase in the size of the much smaller [^{3}H]Ins(3,4,5,6)P_4 pool.

Ins(1,3,4)P_3 would seem to be particularly well suited to its task as an intracellular mediator that links Ins(3,4,5,6)P_4 levels to changes in PLC activity, since cellular levels of Ins(1,3,4)P_3 quite closely follow both the extent and the duration of PLC activation (45, 46). In addition, we have shown the relative ineffectiveness with which the 1-kinase is inhibited by both of the $InsP_2$ products of Ins(1,3,4)P_3 metabolism, namely Ins(1,3)P_2 and Ins(3,4)P_2 (Fig. 7). Thus, the dephosphorylation of Ins(1,3,4)P_3 comprises an efficient signaling off-switch. In this context, it now seems more significant that both the 1- and 4-phosphatases that attack Ins(1,3,4)P_3 may be regulated. This is indicated first by the amino acid sequence of the 4-phosphatase containing consensus motifs for phosphorylation by protein kinases (47). Second, and more directly, Ca^{2+} activates the 1-phosphatase (40).

As a result of this study, we are wiser as to the structural determinants of Ins(1,3,4)P_3-mediated inhibition of the 1-kinase (Table II). We also made the intriguing observation that a 2-phosphate could increase potency of 1-kinase inhibition, depending upon which other phosphate groups were also present. A practical outcome of this increased information concerning specificity is that the non-physiological material, D/L-Ins(1,2,4)P_3, proved to be a particularly potent 1-kinase inhibitor; this could be a productive starting point for the rational design of therapeutically useful drugs that might inhibit the Ins(3,4,5,6)P_4 1-kinase *in vivo*. This provides an alternative to the approach of designing drugs that act at the site of action of Ins(3,4,5,6)P_4 (27).

The very existence of the Ins(1,4,5)P_3 3-kinase, but more so its complex regulation through cross-talk from other signaling pathways, are observations that have been used to bolster the teleological argument that Ins(1,3,4,5)P_4 must be functionally significant (2). Indeed, there is a large body of evidence that Ins(1,3,4,5)P_4 does indeed perform a valuable role inside cells (2). The 3-kinase also has the role of inactivating Ca^{2+} signaling by Ins(1,4,5)P_3. Our new data assign additional significance to this metabolic pathway: control over the production of Ins(1,3,4)P_3, which in turn regulates cellular levels of Ins(3,4,5,6)P_4 (an inhibitor of Ca^{2+}-activated Cl^- channels) (8). The acknowledgment that the Ins(1,4,5)P_3 3-kinase has several important roles provides us with a better appreciation of why so many cellular control processes converge on the regulation of this enzyme's activity (48).

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