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

(Received for publication, March 23, 1999)

Xiaonian Yang‡, Marco Rudolf†, Mark A. Carew‡, Masako Yoshida‡, Volkmar Nerreter‡, Andrew M. Riley‡, Sung-Kee Chung**, Karol S. Bruzik‡‡, Barry V. L. Potter†, Carsten Schultz‡, and Stephen B. Shears‡

From the ‡Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709, the §Institut für Organische Chemie, Universität Bremen, 28359 Bremen, Germany, the ¶Wolfson Laboratories for Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom, the ‡‡Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Chicago, Illinois 60612-7231, **Department of Chemistry, Pohang University of Science and Technology, San 31 Hyoja Dong, Pohang 790-784, Korea

Ca²⁺-activated Cl⁻ channels are inhibited by inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P ₄) (Xie, W., Kaezkel, 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 2,500-fold (yield = 13%) from bovine aorta. Next, we incubated [³H]inositol-labeled AR4–2J cells with cell permeant and non-radiolabeled 2,5,6-tri-O-butyryl-myoinositol 1,3,4-trisphosphate-hexakis(acetoxymethyl) ester. This treatment increased cellular levels of Ins(1,3,4)P ₃ 2.7-fold, while [³H]Ins(3,4,5,6)P ₄ levels increased 2-fold; there were no changes to levels of other ³H-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(1,3,4)P ₂ and Ins(3,4)P ₂, were >100-fold weaker inhibitors of the 1-kinase compared with Ins(3,4,5,6)P ₄ itself (IC₅₀ = 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 (IC₅₀ = 17 and >30 μM, respectively). The non-phosphoryl triphosphates, Ins(1,2,4)P ₃, inhibited 1-kinase relatively potently (IC₅₀ = 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 and Ins(1,3,4,5)P ₄ (Fig. 1) act in a co-ordinated manner as mediators of stimulus-dependent Ca²⁺ mobilization. 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(3,4,5,6)P ₄ 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

* The work in Pohang was supported by the Korean Science and Engineering Foundation/Center for Biofunctional Molecules and Ministry of Education Grant BSRI 97-3437, studies at Bath were supported by Wellcome Trust Program Grant 045491 (to B. V. L. P), and work in Bremen was supported by Deutsche Forschungsgemeinschaft Schu 943/1-6 (to C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 919-541-3308; Fax: 919-541-0559; E-mail: yang@niehs.nih.gov.

§ The abbreviations used are: InsPₙ, inositol polyphosphate, where n is the number of phosphates (e.g. InsP₃); PEG, polyethylene glycol; PLC, phospholipase C; HPLC, high pressure liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; bis-Tris, bis(2-hydroxyethyl)liminotrihydroxymethylmethane; CaM KII, calmodulin-dependent protein kinase; Bt3Ins(1,3,4)P3/AM, 2,5,6-tri-O-butyryl-myoinositol 1,3,4-trisphosphate hexakis (acetoxymethyl) ester.
The Role of Ins(1,3,4)P₃ in Signal Transduction

There was one further aspect to this study that is relevant to the development of Ins(3,4,5,6)P₄ agonists and antagonists for pharmacological intervention in the signaling actions of Ins(3,4,5,6)P₄ (18, 27). This goal is directed at diseases that might be treated by either up-regulating or down-regulating Ca²⁺-activated Cl⁻ secretion (18, 27). A major challenge to pharmacological intervention at the effector site for Ins(3,4,5,6)P₄ comes from the exquisite specificity with which it blocks Cl⁻ channel conductance; Ins(1,3,4)P₃, Ins(1,3,4,5)P₄, Ins(1,3,4,6)P₄, Ins(1,4,5,6)P₄, and Ins(1,4,5,6)P₄ all are ineffective (6, 8, 9). Moreover, at least one, and possibly both of the OH groups on Ins(3,4,5,6)P₄ are 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)P₄. A possible alternative therapeutic strategy might be to target Ins(3,4,5,6)P₄ 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)P₃ that contain a phosphate group in the 2-position.

**Experimental Procedures**

**Materials**—[³H]Insitol was purchased from American Radiolabeled Chemicals Inc. or NEN Life Science Products. [³H]Ins(1,3,4)P₃ was prepared by dephosphorylating [³H]Ins(1,3,4,5)P₄ (20 Ci/mmol, NEN Life Science Products) with recombinant Ins(1,3,4,5)P₄ 5-phosphotase, which was kindly provided by Dr. C. Erneux (29). Ins(1,4,5,6)P₄ was purchased from Sigma. νH-2,5,6-Tri-O-butyryl-myo-inositol 1,3,4-trisphosphate-hexakis(acetoxymethyl) ester (νH-Bt3Ins(1,3,4)P₃/AM; Ref. 19), to elevate the cellular concentration of Ins(3,4,5,6)P₄, is described. The broken line illustrates the metabolic pool of Ins(3,4,5,6)P₄ to steady-state with [³H]inositol.

**Assay of Ins(3,4,5,6)P₄ 1-Kinase**—We have now used AR4–2J cells to examine the functions of inositol polyphosphates in intact cells. We investigated if there was any significant metabolic flux from Ins(1,3,4)P₃ to Ins(3,4,5,6)P₄, which would have revealed itself by tending to decrease the amount of [³H]Ins(3,4,5,6)P₄ 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)P₃, the “on-switch” is dephosphorylation of Ins(1,3,4,5)P₄ (3). This process is particularly sensitive, as Ins(1,3,4,5)P₄ is a 290-fold weaker inhibitor of the 1-kinase than is Ins(1,3,4)P₃ (4). We have now turned our attention to considering how effective is the dephosphorylation of Ins(1,3,4)P₃ as a signaling off-switch. In vivo, both 4- and 1-phosphatases actively degrade Ins(1,3,4)P₃ to Ins(1,3)P₂, and Ins(3,4)P₃ respectively (24–26). We have therefore determined the potency with which these bisphosphate degradation products inhibit the 1-kinase.

where PLC activity was not activated. In this way, we could avoid the possibility of Ins(3,4,5,6)P₄ 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 (Bt3Ins(1,3,4)P₃/AM; Ref. 19), to elevate the cellular concentration of Ins(3,4,5,6)P₄ inside rat pancreatoma (AR4–2J) cells.
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/Ca2+ (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 [3H]Ins(3,4,5,6)P4. In these incubations, 45 pmol of medium containing 67 mM HEPES (pH 8.0 with KOH), 0.7 mM EDTA, 8.7 mM MgSO4, 6.7 mM ATP, 13.3 mM phosphocreatine, 1.33 μM InsP6, 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 [3H]InsP5 formed was determined using gravity-fed ion-exchange columns, as described above.

**Purification of Ins(3,4,5,6)P4 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, 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, diafiltered 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 plus 1 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 III (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 × 56 cm), which was pre-equilibrated with 500 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 B12.

**Culturing and Incubation of AR4–2J Cells**—The AR4–2J pancreatoma cells were cultured in Dulbecco’s modified Eagle’s medium containing 5.4 g/liter glucose, 10% fetal bovine serum, 2 mM glutamine, 500 units/ml penicillin, and 500 μg/ml streptomycin, with 10% condition medium, and harvested by brief trypsinization. Either 2.0 × 107 or 1.2 × 106 cells were seeded in 24-well or 6-well tissue culture plates, respectively. Cells were labeled with 75–150 μCi/ml [3H]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
fractions. The levels of $^3$H-labeled inositol phosphates were normalized as a ratio to cellular levels of $[^3H]$Ins(1,3,4)P$_3$; 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, and 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 $[^3H]$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 $[^3H]$Ins(1,3,4)P$_3$ samples were “spiked” with 4000 dpm $[^3H]$Ins(3,4,5,6)P$_4$ (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 $[^3H]$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 μM 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 $[^3H]$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 $[^3H]$Ins(1,3,4,6)P$_4$ to $[^3H]$Ins(1,3,4,6)P$_4$ that was observed as the Ins(1,3,4,6)P$_4$ 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(1,3,4,6)P$_4$ 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 1800-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 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. 4A). 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-3Ins(1,3,4)P$_3$/AM upon $[^3H]$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$_3$ (Fig. 4, B). 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(1,4,5,6)P$_4$ 3-kinase is a quantitatively minor metabolic route (38, 39), so the ensuing down-stream products, including Ins(1,4,5)P$_3$ are present at unusually low levels in “resting” cells (15). This low base line
The Role of Ins(1,3,4)P3 in Signal Transduction

The potencies with which various inositol phosphates inhibited the Ins(3,4,5,6)P4 1-kinase were determined as described under "Experimental pathway of Ins(1,3,4)P3 dephosphorylation by a 4-phosphatase (39). Control experiments indicated that this lithium medium was therefore supplemented with lithium, so as to inhibit the Ins(1,3,4)P3 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)P3 dephosphorylation by a 4-phosphatase (41). Control experiments indicated that this lithium treatment did not affect levels of [3H]Ins(3,4,5,6)P4 (data not shown).

Extracts of [3H]inositol-labeled control cells were resolved by HPLC, and the various [3H]-labeled inositol phosphates were assayed using an on-line scintillation counting (Fig. 5, upper panel). We also analyzed extracts from cells treated with 200 μM \( \text{D/L-Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \) (Fig. 5, lower panel). In these experiments, any metabolic conversion of Ins(1,3,4)P3 to Ins(3,4,5,6)P4 would, by a pulse-chase effect, tend to decrease the amount of [3H]label in the Ins(3,4,5,6)P4 pool (15). In fact, the opposite result was obtained; the amount of [3H]Ins(3,4,5,6)P4 peaks increased about 2-fold (Fig. 5, Table III). The elevation in [3H]Ins(3,4,5,6)P4 levels in our experiments (Fig. 5) cannot be caused by an increased flux of non-radioabeled Ins(1,3,4)P3 into the [3H]Ins(3,4,5,6)P4 metabolic pool. Note also that there were no significant changes in the sizes of other [3H]inositol-labeled peaks after treatment with \( \text{D/L-Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \). To control for the possibility of nonspecific effects of cell-permeant inositol phosphates, we also incubated AR4–2J cells with 200 μM \( \text{D/L-Bt}_3\text{Ins}(1,3,4,5,6)P_4/\text{AM} \) (which does not affect 1-kinase activity, see below), and this had no effect upon the [3H]inositol-polysphosphate profiles (data not shown).

We were only able to synthesize limited amounts of \( \text{Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \), 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 \( \text{D/L-Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \) approximately doubled the elevation in

**Table II**

| Inositol phosphate       | IC_{50} μM |
|--------------------------|-----------|
| Ins(3,4,5,6)P4           | 1.6 ± 0.8 (4) |
| d,l-Ins(1,3,4,6)P3       | 0.2 ± 0.3 (4) |
| Ins(1,2,3,4,5,6)P5       | 1.5 ± 0.2 (3) |
| Ins(1,3,5,6)P3           | 17 ± 7 (3)   |
| d,l-Ins(1,3,2,4,5,6)P4   | 16.7 ± 4.7 (4) |
| Ins(3,4,5,6)P4           | >30 (3)     |
| Ins(1,4,6)P3            | >30 (3)     |

**Fig. 5. The effect of \( \text{D/L-Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \) upon [3H]inositol phosphates in AR4–2J cells.** [3H]inositol-labeled AR4–2J cells were incubated with \( \text{D/L-Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \) 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, Ins6P; E, Ins(1,4,5,6)P4; F, Ins(1,3,4,6)P4; G, Ins3P; H, InsPc; I, InsPc. The integrated value of the Ins(3,4,5,6)P4 peaks were 640 dpm (upper panel, vehicle) and 1505 dpm (lower panel, \( \text{D/L-Bt}_3\text{Ins}(1,3,4)P_3/\text{AM} \)-treated).

**Fig. 6.** The potencies with which various inositol phosphates inhibited the Ins(3,4,5,6)P4 1-kinase. The potencies with which various inositol phosphates inhibited the Ins(3,4,5,6)P4 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).
We next investigated if the treatment of AR4–2J cells with Bt3Ins(1,3,4)P3/AM elicited increases in levels of Ins(1,3,4)P3 and Ins(3,4,5,6)P4 that were physiologically relevant. For these experiments, we compared the effects of the cell-permeant analog of bombesin with those of receptor-dependent activation of PLC, using bombesin as the agonist. [3H]Insitol-labeled AR4–2J cells were treated for 20 min with 200 nM bombesin, or for 60 min with either 100 μM D-Bt3Ins(1,3,4)P3/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/Bt3Ins(1,3,4)P3/AM used in the experiments described above. In three experiments, 60-min treatment of AR4–2J cells with 100 μM d-Bt3Ins(1,3,4)P3/AM elevated [3H]Ins(3,4,5,6)P4 levels 1.9 ± 0.3-fold (Fig. 6), which is not significantly different from the results obtained with 200 μM d/Bt3Ins(1,3,4)P3/AM (Table III). The fact that these changes in Ins(3,4,5,6)P4 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 [3H]Ins(3,4,5,6)P4 levels (Fig. 6).

We also compared the effects of d-/L-Bt3Ins(1,3,4)P3/AM and bombesin upon cellular Ins(1,3,4)P3 levels (see under “Experimental Procedures”). Stimulation of AR4–2J cells with bombesin elicited a 13-fold increase in levels of Ins(1,3,4)P3 (Fig. 6); treatment with d-Bt3Ins(1,3,4)P3/AM elevated Ins(1,3,4)P3 levels 2.7-fold, an effect that may therefore be considered to be within the physiologically relevant range (Fig. 6).

**Effects of InsP2 isomers on Ins(3,4,5,6)P4 1-kinase Activity**—There is another important issue that is relevant to our evaluation of the significance of Ins(1,3,4)P3 as an intracellular signal. The extent to which metabolites of Ins(1,3,4)P3 also inhibit the 1-kinase contributes to the efficiency with which the cell “switches off” the Ins(1,3,4)P3 signal. The major route of metabolism of Ins(1,3,4)P3 in vivo is its dephosphorylation, by separate 4- and 1-phosphatases, to Ins(1,3)P2 and Ins(3,4)P2, respectively (24–26). We therefore determined the potency with which these bisphosphates degradation products inhibited the 1-kinase, relative to Ins(1,3,4)P3. The IC50 for Ins(1,3,4)P3 was 0.17 μM (Table II and Fig. 4A). In contrast, both Ins(1,5)P2 and d/l-Ins(3,4,5)P3 did not significantly affect 1-kinase activity until their concentration exceeded 3 μM (Fig. 7A); therefore, these bisphosphates were at least 100-fold weaker inhibitors. Thus, dephosphorylation of Ins(1,3,4)P3 is a very effective off-switch for relieving inhibition of 1-kinase activity.

The product of 5-phosphatase attack upon Ins(1,4,5)P3, namely Ins(1,4,1)P3, was found to be a poor inhibitor of the 1-kinase (Fig. 7A). This is also an important observation that demonstrates that it specifically requires Ins(1,4,5)P3 metabolism through the 3-kinase pathway to yield an inhibitor of the Ins(3,4,5,6)P4 1-kinase. In addition, this result shows that all three phosphates of Ins(1,3,4)P3 contribute substantially to the

**TABLE III**

| Table III: The effect of d/Bt3Ins(1,3,4)P3/AM upon levels of [3H]labeled inositol phosphates in AR4–2J cells |
|---------------------------------------------------------------|
| Inositol phosphate | Control | d/Bt3Ins(1,3,4)P3/AM |
|---------------------|---------|---------------------|
| Ins(1,4,5)P3        | 0.049 ± 0.008 | 0.053 ± 0.01 |
| Ins(1,3,4,6)P3      | 0.049 ± 0.008 | 0.048 ± 0.01 |
| Ins(3,4,5,6)P4      | 0.096 ± 0.007 | 0.19 ± 0.006* |
| InsP3               | 4.7 ± 0.49     | 4.5 ± 0.38 |

* indicates the effect of d/Bt3Ins(1,3,4)P3/AM upon the 1-kinase was so effectively imitated by d- nor l-Ins(1,2,4)P3 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)P3 upon the 1-kinase was so effectively imitated by d/l-Ins(1,2,4)P3 (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)P3 as an intracellular signal. First, by using a new, cell-permeant, biactivatable form of Ins(1,3,4)P3, we have shown that Ins(1,3,4)P3 elevates levels of Ins(3,4,5,6)P4 inside intact cells, without Ins(1,3,4)P3 being metabolized to Ins(3,4,5,6)P4. Second, we have discovered that Ins(1,3,4)P3-mediated inhibition of the Ins(3,4,5,6)P4 1-kinase is very efficiently switched off by dephosphorylation of Ins(1,3,4)P3. 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)P4 levels. This is an important issue to resolve, if we are to understand how the cell regulates Ins(3,4,5,6)P4-mediated inhibition of conductance through Ca2+–activated Cl− channels (6, 8). We (15) have previously suggested that Ins(3,4,5,6)P4 levels are controlled by PLC-initiated regulation of the activities of a Ins(3,4,5,6)P4 1-phosphatase/Ins(3,4,5,6)P4 1-kinase substrate cycle (Fig. 1). Others have argued that Ins(1,3,4,6)P4 is converted to Ins(3,4,5,6)P4 by the sequential actions of Ins(1,3,4,6)P4 6-kinase, Ins(1,3,4,6)P4 1-phosphatase, and Ins(3,4,5,6)P4 5-kinase (22, 23, 44). An in-
Bt3Ins(1,3,4)P3/AM enabled us to test this idea.

Pulse of non-radiolabeled Ins(1,3,4)P3 into intact [3H]inositol-

The Role of Ins(1,3,4)P3 in Signal Transduction

creased metabolic flux from Ins(1,3,4)P3 to Ins(3,4,5,6)P4 could raise Ins(3,4,5,6)P4 levels by mass action effects. Bt3Ins(1,3,4,6)P4/AM enabled us to test this idea.

Bt3Ins(1,3,4)P4/AM provided a means of delivering a pulse of non-radiolabeled Ins(1,3,4)P3 into intact [3H]inositol-labeled cells. If, as a consequence of this treatment, there had been a significant metabolic flux from Ins(1,3,4)P3 into the Ins(3,4,5,6)P4 pool, this would have been expected to decrease the [3H]label in this Ins(3,4,5,6)P4 pool by a pulse-chase effect (15). In fact, the opposite result was obtained; Bt3Ins(1,3,4)P4/AM elevated intracellular levels of [3H]Ins(3,4,5,6)P4 (Table III). Thus, for the first time, we have shown that Ins(1,3,4)P4 regulates levels of Ins(3,4,5,6)P4 inside cells, without Ins(1,3,4)P4 being metabolized to Ins(3,4,5,6)P4.

An Ins(1,3,4)P4-mediated inhibition of Ins(3,4,5,6)P4 1-kinase in intact cells would be expected to alter the noise of the substrate cycle that interconverts Ins(3,4,5,6)P4 with Ins(1,3,4,5,6)P5 (Fig. 1). There was not a statistically significant decrease in the levels of [3H]Ins(3,4,5,6)P4 after treatment with Bt3Ins(1,3,4)P4/AM (Table III), but this is not unexpected. The [3H]Ins(3,4,5,6)P4 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 [3H]Ins(1,3,4,5,6)P5 pool.

Ins(1,3,4)P4 would seem to be particularly well suited to its task as an intracellular mediator that links Ins(3,4,5,6)P4 levels to changes in PLC activity, since cellular levels of Ins(1,3,4)P4 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 Ins(3,4,5)P4 products of Ins(1,3,4)P3 metabolism, namely Ins(1,3,4)P3 and Ins(3,4,5,6)P4 (Fig. 7). Thus, the dephosphorylation of Ins(3,4,5,6)P4 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)P4 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, Ca2+ activates the 1-phosphatase (40).

As a result of this study, we are wiser as to the structural determinants of Ins(1,3,4)P3-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, Bt3Ins(1,2,4)P3, 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)P4 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)P4 (27).

The very existence of the Ins(1,4,5)P3 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)P4 must be functionally significant (2). Indeed, there is a large body of evidence that Ins(1,3,4,5)P4 does indeed perform a valuable role inside cells (2). The 3-kinase also has the role of inactivating Ca2+ signaling by Ins(1,4,5)P3. Our new data assign additional significance to this metabolic pathway: control over the production of Ins(3,4,5,6)P4, which in turn regulates cellular levels of Ins(3,4,5,6)P4 (an inhibitor of Ca2+-activated Cl channels) (8). The acknowledgment that the Ins(1,4,5)P3 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).

REFERENCES
1. Berriedge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
2. Irvine, R. F. (1992) in Advances in Second Messenger and Phosphoprotein Research (Putney, J. W., Jr., ed) pp. 161–185, Raven Press, New York
3. Hansen, C. A., Johanson, R. A., Allison, M. T., and Williamson, J. R. (1987) J. Biol. Chem. 262, 17319–17326
4. Tan, Z., Bruzik, K. S., and Shears, S. B. (1997) J. Biol. Chem. 272, 2285–2290
5. Craxton, A., Erneux, C., and Shears, S. B. (1994) J. Biol. Chem. 269, 4337–4342
6. Ho, M. W. Y., Shears, S. B., Bruzik, K. S., Duszyk, M., and French, A. S. (1997) Am. J. Physiol. 272, 1160–1168
7. Vajjanapanich, M., Schultz, C., Rudolf, M. T., Wasserman, M., Eneydi, P., Craxton, A., Shears, S. B., Tsien, R. Y., Barrett, K. E., and Traynor-Kaplan, A. E. (1994) Nature 371, 711–714
8. Xie, W., Kaetted, M. A., Bruzik, K. S., Dedman, J. R., Shears, S. B., and Nelson, D. J. (1996) J. Biol. Chem. 271, 14992–14997
9. Isakovil, i. Fuller, C. M., Berdiev, B. K., Shlyonsky, Y. G., Benos, D. J., and Barrett, K. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10565–10569
10. Nastwong, B. (1998) Am. J. Physiol. 275, G635–G657
11. Petersen, O. H. (1992) J. Physiol. 448, 1–51
12. Large, W. A., and Wang, Q. (1996) Am. J. Physiol. 271, C435–C454
13. Bartel, K. S. (1993) Am. J. Physiol. 265, C599–C606
14. Benntiti, F. S., Oliver, K. G., Nogimori, K., Obie, J. F., Shears, S. B., and Putney, J. W., Jr. (1990) J. Biol. Chem. 265, 11167–11176
15. Craxton, A. K., Putney, J. W., Jr., Obe, J. F., and Shears, S. B. (1992) J. Biol. Chem. 267, 21528–21534
16. Li, W., Schultz, C., Llopis, J., and Tsien, R. Y. (1997) Nature 387, 1207–1204
17. Rudolf, M. T., Wolfson, N., Traynor-Kaplan, A. E., and Schultz, C. (1998) J. Biol. Chem. 273, 19719–19726
18. Rudolf, M. T., Traynor-Kaplan, A. E., and Schultz, C. (1998) Bioorg. Med. Chem. Lett. 8, 1857–1860
19. Shears, S. B. (1989) J. Biol. Chem. 264, 19879–19886
20. Stephens, L. R., Hawkins, P. T., and Downes, C. P. (1989) Biochem. J. 262, 727–737
21. Stephens, L. R., Berrie, C. P., and Irvine, R. F. (1990) Biochem. J. 269, 65–72
22. Stephens, L. R., and Downes, C. P. (1990) Biochem. J. 265, 455–452
The Role of Ins(1,3,4)P$_3$ in Signal Transduction

24. Bansal, V. S., Inhorn, R. C., and Majerus, P. W. (1987) J. Biol. Chem. 262, 9444–9447
25. Inhorn, R. C., Bansal, V. S., and Majerus, P. W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2170–2174
26. Shears, S. B., Kirk, C. J., and Michell, R. H. (1987) Biochem. J. 248, 977–980
27. Shears, S. B., Kirk, C. J., and Michell, R. H. (1987) Biochem. J. 248, 977–980
28. Shears, S. B., Kirk, C. J., and Michell, R. H. (1987) Biochem. J. 248, 977–980
29. Shears, S. B. (1988) Biochim. Biophys. Acta 1436, 49–67
30. Shears, S. B. (1988) Biochim. Biophys. Acta 1436, 49–67