Data from several cell types have indicated that activation of hormone receptors promotes the metabolism of inositol 1,3,4,5,6-pentakisphosphate (IPS) to inositol 3,4,5,6-tetrakisphosphate (3,4,5,6)IP₄. However, to date, metabolism of IPS by cell-free preparations has resulted in the formation of only inositol 1,4,5,6-tetakisphosphate (1,4,5,6)IP₄. Thus, the metabolic relationships of IPS with various inositol tetrakisphosphates (IP₄) isomers have been investigated in both intact cells and cell homogenates of the rat pancreatoma cell line, AR4–2J. The steady-state concentration of IPS was estimated to be 65 nM, while the combined concentration of (3,4,5,6)IP₄ and (1,4,5,6)IP₄ was approximately 1.0 μM. AR4–2J cell homogenates converted (3,4,5,6)IP₄ to (3,4,5,6)IP₄, and (1,4,5,6)IP₄ to IP₃. (1,4,5,6)IP₄, previously has not been demonstrated to be a precursor of IP₃. To alter steady-state levels of inositol phosphates that were maintained by phosphorylation-dephosphorylation cycles, intact cells were treated with 10 μM antimony A which reduced ATP levels by >90% within 10 min. Following 2 h of treatment with antimony A, there was a 6-fold increase in both (3,4,5,6)IP₄ and (1,4,5,6)IP₄, presumably derived from IPS. Experiments with cell-free systems determined that IPS was dephosphorylated to (1,4,5,6)IP₄ by two partially-purified Mg²⁺-independent, Li⁺-insensitive IP₃-3-phosphatase. Moreover, in the presence of 5 mM MgATP, IPS also was metabolized to (3,4,5,6)IP₄. Therefore, our data demonstrate novel and complex relationships between IPS, (3,4,5,6)IP₄, and (1,4,5,6)IP₄.

Studies on the relationship of inositol phosphate metabolism with cellular physiology have centered primarily on the generation and removal of inositol 1,4,5-trisphosphate [(1,4,5)IP₃] and its relation to the regulation of intracellular free Ca²⁺ (1). The pathways and enzymes involved in the catabolic removal of the (1,4,5)IP₃ signal and subsequent salvage of inositol for re-assimilation into inositol lipids have been investigated extensively (2). However, the recent recognition of other inositol phosphates, apparently not directly involved in regulation of intracellular free Ca²⁺, indicates that these inositol phosphates may be involved with other regulatory events in cell physiology. For example, IPS and IP₃ are the most abundant inositol phosphates found in mammalian cells and have been suggested to act as extracellular signalling molecules (3–7) and regulators of aldolase function (8). In spite of these apparent effects, the regulation of intracellular IPS and IP₃ concentrations in intact cells is poorly understood.

Previous studies have indicated that regulation of IPS and IP₃ metabolism may occur in part through phosphorylation-dephosphorylation cycles (9,10). In particular, the hypothesis of an agonist-sensitive IPS 1-phosphatase/(3,4,5,6)IP₄ 1-kinase phosphorylation-dephosphorylation cycle (9) has developed from experiments with intact AR4–2J cells which demonstrated a close metabolic relationship between (3,4,5,6)IP₄ and IP₃ (9) and an apparent agonist-stimulated conversion of IPS to (3,4,5,6)IP₄ (9,11). The agonist sensitivity of this proposed cycle is intriguing and suggests a physiologically significant function. Nevertheless, these proposals would be considerably strengthened if the elements of the putative cycle could be demonstrated in cell-free systems. Although a (3,4,5,6)IP₄ 1-kinase has been observed in cell homogenates (14), there is no direct evidence in cell-free systems for the existence of an IPS 1-phosphatase. In fact, previous studies have reported that cell-free preparations only metabolize IPS to (1,4,5,6)IP₄ (15–17). Therefore, to resolve this observed conflict between IPS metabolism by intact cells and cell homogenates, the synthesis and catabolism of IPS have been investigated in intact AR4–2J cells and AR4–2J cell homogenates. The results indicate that, in unstimulated AR4–2J cells, IP₃ comprises a relatively stable, slowly turning over inositol phosphate pool. Yet under appropriate experimental conditions, interconversion of IP₃ with both (1,4,5,6)IP₄ and (3,4,5,6)IP₄ could be demonstrated in both intact and broken cell preparations. These studies reveal novel pathways of inositol polyphosphate phosphorylation and dephosphorylation and indicate that hormone-regulated interconversion of IPS and various IP₄ isomers may be more complex than previously envisioned.

**EXPERIMENTAL PROCEDURES**

Chemicals—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), dialysed fetal bovine serum, L-glutamine, Puck’s saline A, penicillin/streptomycin, and trypsin/EDTA were purchased from Gibco. [3H]myo-Inositol was purchased from American Radiolabeled Chemicals, St. Louis, MO. [3H]1,3,4,5-IP₃ was obtained from Du Pont-New England Nuclear. Antimycin A was purchased from Calbiochem-Behring Corp. ATP, phosphocreatine, and creatine phosphate were purchased from Sigma. Adsorbosphere SAX HPLC
columns were purchased from Alltech Associates, Deerfield, IL. The Dionex AST column was purchased from Dionex Corp. The Partisphere SAX and Polypropylene FB columns were purchased from Krackeler Scientific, Durham, NC.

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**Cell Culture**—The AR-42J pancreatic cell line was kindly provided by Dr. R. Downes (University of California, San Francisco, and was grown in DMEM containing high glucose (4.5 g/liter), 10% FBS, L-glutamine (2 mM), penicillin/streptomycin (500 units/ml and 500 µg/ml), and 10% conditioned media. Cells were incubated in 6-well tissue culture plates with [3H]myo-inositol (50 µCi/ml) in 2 ml of the above medium with the exception that the FBS was replaced with 10% charcoal-stripped FBS. Cells were cultured with [3H]myo-inositol for 4 days unless otherwise indicated. Cell viability was estimated by trypan blue exclusion.

**[3H]Inositol Phosphate Measurements**—Following culture of AR-42J cells with [3H]myo-inositol, the medium was aspirated and the cells were cultured for an additional 20 min in 1 ml of DMEM + 1% FBS. The medium was again aspirated, and 1 ml of fresh DMEM + 1% FBS either with or without antimycin A was added to the cells for various times. Cells were quenched by the rapid aspiration of the medium followed by the addition of 750 µl of 6% perchloric acid + 250 µg/sample of phytic acid. The plates were incubated at 4 °C for 20 min and the supernatant was removed and neutralized by the freon/trietylamine method of Downes et al. (18) as modified by Shears et al. (19). The [3H]inositol phosphates were eluted from an anion exchange HPLC column (Adsorbosphere SAX, 5-µm particle size) using a linear ammonium phosphate gradient (0-1.0 M; pH 3.5), and the radioactivity was measured on-line as previously described (9, 20). Since (1,4,5,6)IP4, and (3,4,5,6)IP4 are isomers (i.e. L-(3,4,5,6)IP4, and L-(1,4,5,6)IP4, are the same as D-(3,4,5,6)IP4, and D-(3,4,5,6)IP4, respectively), they co-elute on this column. Therefore, unless further structural characterization of this peak is performed, it will be designated D/L-(3,4,5,6)IP4.

**Results**

**Incorporation of [3H]myo-Inositol into IP5 in Intact AR-42J Cells and Synthesis of IP5 by Cell Homogenates**—Investigation of the metabolic relationships of IP5, with its precursors and metabolites was initiated by examining the rate of incorporation of [3H]myo-inositol into various inositol phosphates in intact AR-42J cells and by determining the precursors for IP5 synthesis in cell homogenates. Incubation with [3H]myo-inositol for 1–2 days is required to attain isotope equilibrium for the polyphosphoinositides, (1,4,5)IP3, and its direct metabolites (25, Fig. 1). In contrast, 3–4 days of incubation with [3H]myo-inositol are required for IP5, to attain steady-state incorporation of radioactivity (Fig. 1). Fig. 1 also demonstrates that D/L-(3,4,5,6)IP4, also requires 3–4 days of incubation with [3H]myo-inositol to attain steady-state labeling which is consistent with our previous report closely linking the metabolism of IP5, with D/L-(3,4,5,6)IP4 (9). Following 4 days of incubation with [3H]myo-inositol, there is, compared with (1,4,5)IP3, approximately 32 times more [3H]IP5 and one-half as much [3H]D/L-(3,4,5,6)IP4. Since our previous estimate of basal (1,4,5)IP3 concentration in these cells is 2 µM (29), a minimal estimate for the concentration of IP5 and D/L-(3,4,5,6)IP4 would be 65 µM and 1.0 µM, respectively. Previous analysis of the composition of the IP5 peak in AR4-
2J cells by alkaline hydrolysis indicated the presence of a single IP$_3$ isomer, (1,3,4,5,6)IP$_3$ (9). However, in this study, a more rigorous analysis utilizing two different HPLC elution protocols indicates the additional presence of 0-3% D/L-(1,2,4,5,6)IP$_3$ (data not shown). Our failure to detect D/L-(1,2,4,5,6)IP$_3$ in our earlier studies was due to its low abundance and its co-elution with (1,3,4,5,6)IP$_3$ on Adsorbosphere SAX columns.

IP$_3$ did not attain steady-state incorporation of radioactivity after 4 days of incubation with [³H]myo-inositol, yet at that time there was nearly 9 times more [³H]IP$_4$ than [³H]IP$_3$ (1,4,5)IP$_3$ resulting in a minimal concentration of 17 μM for IP$_3$. These data indicate that in unstimulated cells IP$_3$ and IP$_4$ constitute large, slowly turning over pools of inositol phosphates which are kinetically far removed from the polyphosphoinositides. Similar conclusions have been reached previously by using different experimental protocols (9, 30).

Previously, differential labeling kinetics in intact AR4–2J cells (9) and phosphorylation data from various cell homogenates (14, 16, 17, 26, 31) have indicated that both (1,3,4,6)IP$_4$ and (3,4,5,6)IP$_4$ may be utilized as precursors for IP$_4$. In agreement with these results, Fig. 2 demonstrates that AR4–2J cell homogenates are capable of converting both (3,4,5,6)IP$_4$ (panel A) and (1,3,4,6)IP$_4$ (panel B) to IP$_3$. In addition, panel C demonstrates for the first time that (1,4,5,6)IP$_4$ can be converted to IP$_3$. HPLC analysis indicated that each IP$_3$ isomer was phosphorylated only to (1,3,4,5,6)IP$_5$ (data not shown). Therefore, one IP$_3$ isomer, (1,3,4,6)IP$_4$, which is derived from a pathway initiated by polyphosphoinositide hydrolysis, and two IP$_4$ isomers, (3,4,5,6)IP$_4$ and (1,4,5,6)IP$_4$, whose syntheses apparently are not closely linked to the polyphosphoinositides, are capable of contributing to the formation of IP$_4$.

**IP$_3$ Metabolism in Intact AR4–2J Cells**—Antimycin A rapidly reduces cellular ATP levels and greatly reduces the levels of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, thereby demonstrating the rapid turnover rate of the polyphosphoinositides (32, 33). With these results in mind, the effect of antimycin A treatment on IP$_3$, IP$_4$, and IP$_5$ isomers has been studied in intact AR4–2J cells. Since multiple IP$_3$ isomers and IP$_4$ may be associated with IP$_5$ through phosphorylation-dephosphorylation cycles (9, 10), lowering ATP levels should perturb the phosphorylation pathways within this cycle and reveal precursors and pathways of IP$_4$ metabolism.

**Antimycin A treatment of AR4–2J cells reduces ATP levels by >90% within 10 min (33).** As shown in Fig. 3, antimycin A treatment for 1 h resulted in a 4-fold increase in a single IP$_4$ peak, D/L-(3,4,5,6)IP$_4$ (peak C). This increase was significant within 10 min after antimycin A treatment as compared to zero time basal levels and increased to greater than 6-fold over basal levels by 2 h (Fig. 4). We believe that this increase in D/L-(3,4,5,6)IP$_4$ from 1.0 μM (see above) to 6 μM was due to an antimycin A-mediated decrease in the rate of flux from IP$_3$ to IP$_4$ (see "Discussion"). A corresponding decrease in IP$_4$ levels (i.e. 5 μM) was observed but, due to the high level of IP$_3$, this proportionately small decrease was not statistically significant.

The D/L-(3,4,5,6)IP$_4$ peak obtained from antimycin A-treated cells was structurally characterized to distinguish between (1,4,5,6)IP$_4$ and (3,4,5,6)IP$_4$. This was accomplished through a multistep chiral analysis, the final step of which involves oxidation of L-[³H]iditol by a stereospecific dehydrogenase. The amount of (3,4,5,6)IP$_4$ in a mixture of D/L-[³H]iditol (9) is identified from the oxidation of its L-[³H]iditol derivative. The basal composition of D/L-(3,4,5,6)IP$_4$ in AR4–2J cells consisted of approximately equal amounts of (1,4,5,6)IP$_4$ and (3,4,5,6)IP$_4$ (9) (data not shown). Following antimycin A treatment for 1 h (4-fold increase in D/L-(3,4,5,6)IP$_4$), approximately 60% of this peak was (3,4,5,6)IP$_4$ (Fig. 5), the balance being (1,4,5,6)IP$_4$. These effects of antimycin A appeared to be due solely to depletion of cellular ATP since the addition of 10 μM antimycin A to cell homogenates did not affect either the rate of phosphorylation of (1,4,5,6)IP$_4$ and (3,4,5,6)IP$_4$ or the rate of IP$_3$ dephosphorylation.
Antimycin A induces a similar increase in the levels of both 
Cation (data not shown). Thus, by decreasing ATP levels, 
antimycin A was oxidized, approximately 60% of the unknown D/L-[3H]iditol was 
was oxidized, reduced, and dephosphorylated and chromato-
standard D-[1-3H]iditol was incubated with the L-iditol-specific dehy-
graphed on a Polybore-PB column, and the D/L-[3H]iditol peak was 
1 h. Inositol phosphates were prepared for structural characterization 
in AR4–2J cells. AR4–2J cells were labeled for 4 days with [3H]
mvo-inositol. The cells were then incubated with DMEM + 1% FBS in the absence (closed symbols) or the presence (open symbols) of 
antimycin A (10 μM, stock 10 mM in ethanol) for various times. The 
inositol phosphates were resolved by HPLC. There was no significant 
change in the level of IP₃ when expressed relative to either phos-
phoinositide levels or cellular protein. Therefore, the amounts of D/ 
L-(3,4,5,6)IP₄ (squares) and IP₅ (circles) are expressed relative to the 
IP₃ level. Error bars are not displayed when smaller than the symbols. 
Shown are the mean ± S.E. from triplicate determinations in five 
experiments in the presence of antimycin A, and triplicate determinations 
in two concurrently performed experiments in the absence of 
antimycin A. * p < 0.05 by two-tailed unpaired t test. Since levels of 
D/L-(3,4,5,6)IP₃ did not change in the absence of antimycin A, 
statistical significance was tested relative to zero time controls.

Structural analysis of the IP₃ peak following up to 2 h of 
antimycin A treatment demonstrated that the primary IP₃ isomer present was (1,3,4,5,6)IP₃ (97%) and that there was no change in the small amount of D/L-(1,2,4,5,6)IP₃ present (data not shown). The lack of change in IP₅ levels may indicate that futile cycles between IP₃ and IP₅, which have been demonstrated in Dictyostelium (10), do not exist or proceed very slowly in mammalian cells. Therefore, the rapid depletion of the polyphosphoinositides (32, 33), compared to the minimal effect on levels of IP₃ and IP₅ following antimycin A treatment, further supports our above conclusion that IP₃ and IP₅ exist as pools of inositol phosphates which are independent and kinetically distinguishable from the inositol lipids.

Antimycin A treatment induces two additional changes in the observed basal pattern of inositol phosphates in AR4–2J cells. Firstly, in Fig. 3 there is a [3H]-labeled peak present in basal AR4–2J cells that is more polar than IP₃. A peak eluting after IP₃ also has been noted in Dictyostelium (34). While this peak is depleted by 1 h of antimycin A treatment (Fig. 3), other experiments (not shown) demonstrated that it had an approximate half-life of 5 min. Thus, this compound apparently has a rapid turnover rate. Secondly, after 1 h of antimycin A treatment, an additional IP₅ peak occasionally is observed (<10% of HPLC separations) that elutes less than 1 min after (1,4,5)IP₃. Our failure to reproducibly observe this IP₅ isomer is most likely a result of the inability of our HPLC system to consistently separate this peak from (1,4,5)IP₃. This is supported by our previous observation of a similarly eluting IP₅ peak in basal AR4–2J cells that was revealed only after removal of (1,4,5)IP₃ by treatment with purified 5-phosphatase (35). Structural characterization of this peak would be useful but presently is hampered by its low levels.

F[3H]IP₃, Dephosphorylation by AR4–2J Cell Homogenates—The rate of IP₃ hydrolysis by cell homogenates was compared to that by 100,000 × g particulate and soluble fractions. The relatively high level of IP₃ in AR4–2J cells could contribute significant and variable amounts of IP₃ to our incubations (up to 0.4 μM)². Therefore, to minimize this potential variability in IP₃ amounts, nonradioactive IP₃ was added to the [3H]IP₃ to result in a final concentration of 10 μM. This maneuver should also reduce the ability of endogenous IP₃ (0.1 μM)² to competitively inhibit IP₃ dephosphorylation (36). Incubation of either AR4–2J cell homogenates, 100,000 × g pellet, or 100,000 × g supernatant with [3H]IP₃ resulted in the appearance of a major IP₃ peak eluting in the position of 
D/L(3,4,5,6)IP₃ (data not shown). Structural analysis determined that (1,4,5,6)IP₃ was the only detectable IP₃ isomer produced from [3H]IP₃ dephosphorylation by AR4–2J cells. Therefore, in agreement with other cell types, AR4–2J cells possess an IP₃ 3-phosphatase activity (12, 15–17, 36). The majority of this IP₃ 3-phosphatase activity was associated with the membrane fraction. When expressed relative to total cellular protein, the fractions contained (pmol/mg of protein/min): homogenate, 75 ± 18; membrane fraction, 46 ± 3; soluble fraction, 14 ± 7; intact cells, 12 ± 2 (means ± S.E., n = 3). Unlike NIH-3T3 cells (37), AR4–2J cells exhibited little or no extracellular IP₃ phosphatase activity on their surface, once the presence of nonviable, permeable AR4–2J cells (5–10% by trypan blue exclusion) was taken into account.

Further experiments to characterize this IP₃ 3-phosphatase 
²The protein concentration used (1.0 mg/ml) results in an approximate 166-fold dilution of the cytoplasm (volume of intact AR4–2J cells is approximately 6 μl/mg of protein (29)); thus, the contributions of endogenous IP₃ and IP₅ would be approximately 0.4 μM and 0.1 μM, respectively.
activity revealed that it was not inhibited by Li⁺ (20 mM) or by removal of Mg²⁺, and it was only activated by Ca²⁺ at concentrations above 500 μM (data not shown). The dephosphorylation rate of the IP₃ 3-phosphatase was pH-dependent, was nearly tripled under alkaline conditions (pH 8.5), and was diminished by one-half under acidic conditions (pH 5.5) as compared to neutral conditions (pH 7.2). Even under these different pH conditions, the only IP₄ detected was the (1,4,5,6)IP₄ isomer (data not shown).

**Effect of ATP on the IP₄ Products of IP₃ Dephosphorylation**—In these experiments, [³H]IP₃ was incubated with AR4–2J cell homogenates in the presence or absence of 5 mM MgATP. Since no nonradioactive IP₃ was added in these incubations, the starting concentration of IP₃ was approximately 0.4 μM due to the contribution of endogenous IP₃ from the cell homogenates. Following various times of incubation, structural analysis was done on any D/L-(3,4,5,6)IP₄ formed.

After incubation of AR4–2J cell homogenates with [³H]IP₃ for 30 min in the absence of MgATP, 8% of the [³H]IP₃ was metabolized, and the D/L-(3,4,5,6)IP₄ was found to be entirely (1,4,5,6)IP₄ (i.e. no L-[³H]iditol was detected, Fig. 6). Thus, reducing the IP₃ concentration from 10 μM (as in the experiments above) to approximately 0.4 μM did not affect the composition of the D/L-(3,4,5,6)IP₄ peak. In contrast, when IP₃ was incubated for 30 min in the presence of 5 mM MgATP, 11% of the IP₃ was metabolized and 17% of the total D/L-(3,4,5,6)IP₄ peak was (3,4,5,6)IP₄ (Fig. 6). Under these conditions, less than 2% of the total radioactivity was recovered as inositol phosphates other than D/L-(³H)(3,4,5,6)IP₄, or [³H]IP₅. This experiment represents the first observation that IP₄ can be converted to (3,4,5,6)IP₄ in a cell-free system. Therefore, the kinetics of this process were studied in more detail (Fig. 7). In addition to being an absolute requirement for the accumulation of (3,4,5,6)IP₄, the inclusion of ATP increased the yield of (1,4,5,6)IP₄ (63% increase after 60-min incubations) by a mechanism which is not clear. The kinetics of appearance of (3,4,5,6)IP₄ indicated that at the earliest time point analyzed (10 min), (3,4,5,6)IP₄ was present at approximately one-third the level of (1,4,5,6)IP₄. However, by 60 min of incubation, the (3,4,5,6)IP₄ level was only 10% of the (1,4,5,6)IP₄.

One possible explanation for the ATP requirement to observe (3,4,5,6)IP₄ may be due to an ATP-mediated inhibition of a (3,4,5,6)IP₄ phosphatase. Therefore, we examined the effect of 5 mM MgATP upon (3,4,5,6)IP₄ dephosphorylation in 30-min incubations that were identical with those used for studying IP₄ dephosphorylation. In the absence of ATP, only 11% of the total (3,4,5,6)IP₄ was dephosphorylated (data not shown). The inclusion of MgATP reduced (3,4,5,6)IP₄ dephosphorylation to 1–2% (data not shown). Therefore, if (3,4,5,6)IP₄ had been formed from IP₅ in the absence of ATP, then 89% of the (3,4,5,6)IP₄ would have been detected. The presence of ATP would have been expected to increase the accumulation of any (3,4,5,6)IP₄ to 98%. Thus, during IP₃ dephosphorylation, the addition of MgATP would be predicted to increase the accumulation of any (3,4,5,6)IP₄ by only a factor of 1.1, if it was acting solely to inhibit (3,4,5,6)IP₄ dephosphorylation. Since the effect of MgATP on IP₄ conversion to (3,4,5,6)IP₄ is far more dramatic (Figs. 6 and 7), we conclude that the nucleotide is acting in a substantially different manner (see “Discussion”). When the nonhydrolyzable ATP analogue, AMP-PNP, was incubated with [³H]IP₃ and AR4–2J cell homogenates, no (3,4,5,6)IP₄ was formed (data not shown). This result implies that conversion of IP₃ to (3,4,5,6)IP₄ requires hydrolysis of ATP. AMP-PNP also failed...
to imitate the stimulatory effect of ATP on the rate of conversion of IP₃ to (1,4,5,6)IP₄ in incubations of up to 60 min (data not shown).

**DISCUSSION**

This study has uncovered novel precursor/product relationships between IP₃ and several IP₄ isomers. By utilizing IP₄ isomers normally present in AR4-2J cells as potential precursors for IP₃ synthesis, we confirmed findings in other cell types that either (1,3,4,6)IP₄ or (3,4,5,6)IP₄ can be converted to IP₃ (9, 14, 16, 17, 26, 31). In addition, for the first time, (1,4,5,6)IP₄ was shown to be converted to IP₃. Therefore, at least three different IP₃ isomers can function as precursors for the synthesis of IP₄.

Determination of the involvement of these three IP₃ isomers in phosphorylation-dephosphorylation cycles in intact cells was approached by treatment with antimycin A. Lowering of cellular ATP levels to inhibit phosphorylation and thus expose dephosphorylation reactions resulted in an increase in both (3,4,5,6)IP₄ and (1,4,5,6)IP₄. Since no agonist activation is involved, these changes presumably reflect processes involved in maintaining equilibrium levels of IP₃ and p/-(3,4,5,6)IP₃ in these cells. This indicates that both the previously proposed agonist-sensitive (3,4,5,6)IP₃-IP₄ cycle (9) and a novel (1,4,5,6)IP₃-IP₄ cycle may exist in intact AR4-2J cells.

In addition to the phosphorylation of both (3,4,5,6)IP₄ and (1,4,5,6)IP₄ to IP₃, it was also possible to demonstrate for the first time conversion of IP₃ to both of these isomers by broken cell preparations. However, (3,4,5,6)IP₃ could only be produced in the presence of ATP. This requirement for ATP provides an explanation for the failure of earlier studies to detect this reaction (15, 16). Mattingly et al. (17) also have studied IP₃ dephosphorylation in the presence of ATP, but under their assay conditions only (1,4,5,6)IP₃ was detected. A potential explanation for the difference in their results and ours is that their experiments were performed with cytosolic fractions, and our experiments were performed with homogenates. This implies that the putative 1-phosphatase may be exclusively a particulate enzyme or alternatively that there may be a factor present in particulate fractions that is necessary for the formation of (3,4,5,6)IP₄. Additionally, the utilization of a different cell type in our experiments may account for our different results.

The ATP dependence for conversion of IP₃ to (3,4,5,6)IP₄ and the phosphorylation of (1,4,5,6)IP₃ suggests at least two possible models for the metabolic relationships of IP₃ with (1,4,5,6)IP₃ and (3,4,5,6)IP₄ (Fig. 8). One model (Fig. 8, top) consists of two independent phosphorylation-dephosphorylation cycles between IP₃ and either (1,4,5,6)IP₄ or (3,4,5,6)IP₄. This model supports the following predictions. Firstly, agonist activation perturbs the equilibrium between (3,4,5,6)IP₄ and IP₃ either by enhancing IP₃ 1-phosphatase activity and/or by inhibiting (3,4,5,6)IP₄ 1-kinase activity. Secondly, the IP₃ 1-phosphatase activity requires ATP. However, since antimycin A treatment results in an increase in (3,4,5,6)IP₄, the Kᵣ for ATP activation of the IP₃ 1-phosphatase would have to be lower than that for the (3,4,5,6)IP₄ 1-kinase. Thus, the ATP requirement for IP₃ 1-phosphatase activity may provide a potentially important control point in this model. The inability of AMP-PNP to mimic the ATP requirement indicates that ATP hydrolysis is required, which could indicate that the IP₃ 1-phosphatase is activated by phosphorylation. Therefore, the ability of agonist activation to increase the amount of (3,4,5,6)IP₄ may be through agonist activation of this phosphorylation.

An alternative model, which also is consistent with our data, involves only one cycle between (1,4,5,6)IP₃, (3,4,5,6)IP₄, and IP₃ (Fig. 8, bottom). The cycle would be initiated by dephosphorylation of IP₃ to (1,4,5,6)IP₄, followed by the conversion of (1,4,5,6)IP₄ to (3,4,5,6)IP₄. This latter reaction could occur through either an ATP-dependent isomerase reaction or by dephosphorylation to (4,5,6)IP₃ and subsequent phosphorylation to (3,4,5,6)IP₄. Phosphorylation of (3,4,5,6)IP₄ to IP₃ would then complete the cycle. According to this model, agonist activation promotes the formation of (3,4,5,6)IP₄, by one or more of the following ways: 1) enhancing the rate of IP₃ dephosphorylation to (1,4,5,6)IP₄, 2) enhancing the conversion rate of (1,4,5,6)IP₄ to (3,4,5,6)IP₄, or 3) inhibiting the phosphorylation of (3,4,5,6)IP₄ to IP₃. A second prediction of this model is that antimycin A-mediated decreases in ATP levels would more greatly decrease (3,4,5,6)IP₄ phosphorylation to IP₃ than it would (1,4,5,6)IP₄ conversion to (3,4,5,6)IP₄. Although the presence of (4,5,6)IP₄ has not been confirmed, small amounts of an unknown IP₄ isomer have been observed in two instances: in basal AR4-2J cells (36) and occasionally in AR4-2J cells following antimycin A treatment (see “Results”). If the conversion of (1,4,5,6)IP₃ to (3,4,5,6)IP₄ is rapid or occurs through a multienzyme complex, then only small amounts of this IP₄ intermediate may be seen. Regardless of the actual pathway that exists in AR4-2J cells, our data demonstrate that IP₃ participates in metabolic cycles with at least two different IP₄ isomers.

The ability of different physiological changes within the cell (v-src transformation (17), chronic agonist stimulation (9, 12, 13), and cell differentiation (38)) to perturb either IP₃ concentrations or individual IP₄-IP₃ cycles indicates that, in addition to the more traditional agonist-sensitive inositol phosphates (1,4,5,6)IP₃ and its metabolites), IP₃ and its metabolites also may be involved in regulation of physiological processes. Theoretically, flux through IP₃-IP₄ cycles may be differentially regulated by a variety of agonists in order to elicit individual cellular responses (38). Thus, the complex regulation of IP₃ metabolism may be analogous to the inositol lipid pool which can act as a substrate for phospholipase C to form (1,4,5,6)IP₃ and also can be phosphorylated by a 3-kinase to form a less well characterized pool of inositol lipids that may be important in growth factor action or cell transformation (39). In addition to the well characterized rapid effects of (1,4,5,6)IP₃ on cellular Ca²⁺ metabolism, it seems likely that other important, albeit more subtle, cellular functions may be controlled through the regulated metabolism of IP₃ to specific
inositol tetrakisphosphate isomers.

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