Origins of \textit{myo}-inositol Tetrakisphosphates in Agonist-stimulated Rat Pancreatoma Cells

STIMULATION BY BOMBESIN OF \textit{MYO-INOSITOL 1,3,4,5,6-PENTAKISPHOSPHATE BREAKDOWN TO MYO-INOSITOL 3,4,5,6-TETRAKISPHOSPHATE}* 

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In the rat pancreatoma cell line, AR4-2J, three inositol tetrakisphosphate isomers were identified, (1,3,4,6), (1,3,4,5), (3,4,5,6), which were increased during activation of phospholipase C by bombesin. Two other isomers were identified, (1,4,5,6) and a fifth isomer which was either (1,2,3,4) or (1,2,3,6), which have not previously been detected in any cell type. To study the metabolic interrelationships between these compounds and inositol 1,3,4,5,6-pentakisphosphate in the intact cell, their turnover was assessed under different protocols of [\textsuperscript{3}H]\textit{myo}-inositol labeling; the inositol phosphates were labeled to near steady state or under conditions where either rapidly or slowly turning over inositol polyphosphates were preferentially labeled. The relative specific radioactivities of inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,6-tetrakisphosphate were very similar in bombesin-stimulated cells, consistent with the pathway for the conversion of inositol 1,4,5-trisphosphate to the other three inositol polyphosphates. Compared with these inositol phosphates, the turnover of inositol 1,3,4,5,6-pentakisphosphate was slow. An accumulation of radioactivity into inositol 1,3,4,5,6-pentakisphosphate was observed only under labeling conditions where its relative specific radioactivity was substantially below that of inositol 1,3,4,6-tetrakisphosphate. This indicated that the precursor for \textit{de novo} synthesis of inositol 1,3,4,5,6-pentakisphosphate was inositol 1,3,4,5,6-tetrakisphosphate. Bombesin stimulated the net breakdown of inositol 1,3,4,5,6-pentakisphosphate and increased the level of inositol 3,4,5,6-tetrakisphosphate; the relative specific radioactivities of these two compounds were similar under all conditions. These data led to the novel proposal that inositol 3,4,5,6-tetrakisphosphate is the product of inositol 1,3,4,5,6-pentakisphosphate breakdown. This reaction was apparently stimulated by a regulated change in the enzyme(s) which interconvert inositol 1,3,4,5,6-pentakisphosphate and inositol 3,4,5,6-tetrakisphosphate.

It is now appreciated that the formation and metabolism of inositol polyphosphates is a complex process which occurs in a wide variety of cell types (1, 2). The most well-characterized step in this process is the formation of inositol 1,4,5-trisphosphate (1(1,4,5)IP_{3}), which results from the cell surface receptor-activated breakdown of the membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_{2}), catalyzed by phospholipase C. The function of (1,4,5)IP_{3} has been defined in mammalian cells; it is the intracellular messenger which initiates the process of Ca\textsuperscript{2+} mobilization (3). (1,4,5)IP_{3} is metabolized by dephosphorylation to (1,4)IP_{2} (4) and by phosphorylation to (1,3,4,5)IP_{4} (5). Dephosphorylation clearly serves to inactivate (1,4,5)IP_{3} since (1,4)IP_{2} does not release intracellular Ca\textsuperscript{2+} stores. However, it has been suggested that (1,3,4,5)IP_{4} has a distinct intracellular function, namely, to augment (1,4,5)IP_{3}-induced Ca\textsuperscript{2+} mobilization (2). Furthermore, (1,3,4,5)IP_{4} is but one of several highly phosphorylated inositol phosphates, including at least three additional inositol tetrakisphosphates ((1,4,5,6)IP_{4}, (3,4,5,6)IP_{4}, and (1,3,4,6)IP_{4}, (6, 7)), which may increase upon receptor stimulation. This raises the possibility that the inositol polyphosphates comprise a family of intracellular messengers which serve to regulate intracellular Ca\textsuperscript{2+} as well as other, as yet undefined, cellular processes. Given this premise, it is important to understand the metabolic interrelationships among the inositol polyphosphates, particularly during stimulation of phospholipase C activity.

In the present study, we have examined the effects of agonist activation on inositol tetrakis- and pentakisphosphate metabolism in the rat pancreatoma cell line, AR4-2J. To gain insight into these metabolic interrelationships in the intact cell, we have expanded the technique of measuring the accumulation and disappearance of [\textsuperscript{3}H]inositol polyphosphates by using different protocols for labeling the cells with [\textsuperscript{3}H]myo-inositol. In one condition, the inositol phosphates were labeled to near steady state, whereas in other conditions either rapidly or slowly turning over inositol polyphosphates were preferentially labeled. Thus, through comparison of relative specific radioactivities of the [\textsuperscript{3}H]inositol lipids and [\textsuperscript{3}H]inositol polyphosphates across these labeling conditions, specific metabolic relationships among the inositol polyphosphates were revealed. Our findings indicate that agonist activation causes an accumulation of three inositol tetrakisphosphates in the AR4-2J cells. Two of these, (1,3,4,5)IP_{4} and (1,3,4,6)IP_{4}...  

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1 The abbreviations used are: The inositol phosphates and lipids are abbreviated according to the "Chilton Convention" (34) as, for example, (1,4,5)IP_{3} for \textit{d-myо}-inositol (1,4,5)trisphosphate and PIP_{2} for phosphatidylinositol (4,5)bisphosphate; HEPES, 4-(2-hydroxy-ethyl)-l-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; HPLC, high performance liquid chromatography.
(1,3,4,6)IP₄ are derived from the metabolism of the phospholipase C product, (1,4,5)IP₃. Surprisingly, the third inositol tetrakisphosphate, (3,4,5,6)IP₄, appears to arise by an independent pathway involving agonist-induced breakdown of (1,3,4,5,6)IP₄.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Dulbecco's modified Eagle's medium, qualified fetal bovine serum, glutamine, and Puck's saline A were from GIBCO. Bombesin was from Peninsula Laboratories, Inc. (Belmont, CA). Antibiotics (penicillin-streptomycin) and agarose, 1% w/v, were from Difco. (1,4,5,6)IP₄, (1,3,4,6)IP₄, and (1,4,5,6)IP₃, were from from BioRad, (1,3,4,5)IP₃ was from New England Nuclear. (1,3,4,5,6)IP₄, (1,3,4,5,6)IP₃, (1,3,5,6)IP₃, and (1,3,4,6)IP₃ were from Amersham Corp. (1,3,4,5)IP₃, (1,3,4,5,6)IP₄, and (1,3,4,5,6)IP₃ were prepared as described by Shears et al. (9). [3H]myo-Inositol (6-10 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO) or New England Nuclear (E. I. Du Pont de Nemours & Co., Inc.). (1,3,4,5)IP₃, [3H](1,4,5)IP₃, and [3'P](1,4,5)IP₃ were from New England Nuclear. (32P)IP₃ was generously donated by Dr. L. R. Stephens (Dept. of Biochemistry, University of California, San Francisco, CA). 

**Inositol—**Inositol lipids were extracted from the cell matrix as described below. [3H]Inositol lipids were extracted from the cell matrix as described below. 

**Preparation of Perchloeric Acid Supernatants—**Perchloeric acid supernatants were prepared as described below.

**Inositol Phosphate Assay—**Supernatants from perchloeric acid-stoppage reactions were neutralized by the tris/tri-n-acylamylamine method of Downes et al. (15) as modified by Shears et al. (16). Identification of [3H]inositol phosphates from this extraction procedure was presented in the figures; within each experiment, each samples were separated on an anion exchange column (Adsorbosphere SCX-8, 5-µm particle size, Alltech Associates, Inc., Deerfield, IL) eluted with an ammonium phosphate gradient according to the method of Balla et al. (16, 17, 18, see Fig. 1).

**Alkaline Hydrolysis of Inositol Tetrakisphosphates—**Alkaline hy-

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K. O. Oliver, F. S. Memmit, and J. W. Putney, Jr., unpublished observations.
The mixture of inositol polyphosphates produced is indicative of the structure of the parent compound (11, 19). This procedure was performed as described in detail elsewhere (11, 19). The resultant [3H]inositol monophosphates, and added [3C]- or [3P]inositol monophosphate standards, were separated by HPLC as described by Stephens et al. (19) with the following modifications: the eluting gradient was constructed from water (solvent A) and 0.2 M sodium acetate (pH 3.75 with acetic acid, solvent B): 0–10 min, B = 0%; 10–11 min, B increased linearly to 30%; 11–120 min, B = 30%; 120–134 min, B increased linearly to 100%; 134–144 min, B = 100%. The flow rate was 1 ml/min. Fractions of 0.6 ml were collected and radioactivity determined by dual label liquid scintillation counting. Note that this technique does not separate enantiomers, thus (1)IP and (3)IP coelute, as do (4)IP and (6)IP.

Periodate Treatment of Inositol Tetrakisphosphates—Inositol polyphosphates are oxidized at vicinal hydroxyls upon incubation with periodate. The polyp formed subsequent to reduction and dephosphorylation is dependent on the parent inositol tetrakisphosphate. [3H]inositol tetrakisphosphate peaks collected from cell samples were incubated with periodate, reduced, and dephosphorylated according to procedures described elsewhere (11, 12). The [3H]polys were separated by HPLC using a Polybore Pb column as previously described (12, 19) except that a titanium pump and injector (Pharmacia LKB Biotechnology, Inc.) were employed, and 0.4-ml fractions were collected. The polyp identity was determined from the elution of [3C]- and [3H]-labeled standards (see "Results"). In some experiments, individual [3H]polyp peaks eluting from the Polybore Pb column were collected, lyophilized, and rechromatographed on an Aminex HPX-87H column (Bio-Rad) eluting at 30°C at a flow rate of 0.2 ml/min and 0.2 ml fractions were collected. Typical elution times from this column were (in min): inositol, 27; altritol, 28.5; iditol, 30.

Polyol Dehydrogenase Assay—Following periodate oxidation, reduction, and dephosphorylation, [3H](1,3,4,5)IP, and [3H](1,4,5,6)IP yield L- and n-[3H]iditol, respectively. To distinguish these enantiomers, [3H]iditol isolated by HPLC as described above was lyophilized and incubated with a polyol dehydrogenase (EC 1.1.1.14) which preferentially oxidizes L-iditol to sorbose (at the expense of NAD, 19). The procedure was identical to that described elsewhere (19), except that the concentration of internal standard of L-iditol was 100 μM, and the incubations were for 30 min at 30°C. The reaction was monitored by following the increase in absorbance at 340 nm due to the NADH formed, and hence, the extent of oxidation of non-radioactive L-iditol. In these incubations, 80% of the L-iditol was converted to sorbose whereas, in parallel experiments, less than 5% of n-[3H]iditol standard was oxidized (data not shown). The incubations were quenched by adding ice-cold 0.6 M perchloric acid plus 0.3 mg/ml exchange columns (1 ml, AG 1-X8, formate form). The columns were using dual label liquid scintillation counting techniques.

Identity of Inositol Polyphosphates in AR4-2J Cells—In AR4-2J cells labeled for 72 h with [3H]myo-inositol, various [3H]inositol polyphosphates accumulated (Fig. 1). Two inositol trisphosphates were identified. In both unstimulated cells and cells stimulated for 60 min with a maximally effective concentration of bombesin (200 nM), greater than 95% of the radioactivity eluting in the peak ascribed to [3H](1,4,5)IP was authentic (1,4,5)IP since this material was found to be virtually indistinguishable from [3P](1,4,5)P3 standard as an in vitro substrate for both (1,4,5fIP3 3-kinase and (1,4,5)IP3 5-phosphatase. The [3H](1,3,4,5)IP3 peak is so ascribed based on its coelution with [3H](1,3,4,5)IP3 standard (data not shown).

Three peaks were observed in the inositol tetrakisphosphate region of the chromatogram (Fig. 1). Based on its retention time relative to an internal standard of [32P](1,3,4,5)P4 (data not shown), the first eluting peak was initially believed to be [3H](1,3,4,6)IP3 (17, 18). Alkaline hydrolysis of this material yielded the expected [3H] labeled (1)IP(3,IP plus (4)IP(6)IP. However, a small proportion of [3H](2)IP was also recovered (Fig. 2). The [3H](1,3,4,5)IP3 peak accounted for 7 ± 2% (three experiments) of the total [3H]monophosphates derived from samples prepared from unstimulated cells. This proportion decreased to 1.5 ± 0.5% (three experiments) in samples from stimulated cells (Fig. 2). That the first eluting inositol tetrakisphosphate peak contained at least two constituents was confirmed by subjecting the [3H]-labeled material in this peak to incubation with periodate, reduction, and dephosphorylation. Two [3H]-

Inositol Tetrakisphosphates in AR4-2J Cells

Fig. 2. Inositol monophosphates produced by alkaline hydrolysis of the first eluting inositol tetrakisphosphate peak. The first eluting [3H]inositol tetrakisphosphate peak from unstimulated (upper panel) or bombesin (BBS)-stimulated (lower panel) cells was subjected to alkaline hydrolysis, and the resultant [3H]inositol monophosphates (solid lines) were determined by HPLC as described under "Experimental Procedures." Elution times varied between samples, but the identity of each peak was retained from retention times relative to internal standards of [3H]IP3, [3H]IP2, and [3H]IP (6)IP (dotted lines). Data are expressed as the percentage in each fraction of the total [3H]-labeled material or [3H]IP and [3H]IP2 labeled standards applied to the column. In this experiment, the percentages of the total [3H]-labeled material in the peaks were relative to stimulated cells: 1(IP)/(BIP) 19%, 2(IP) 2%, 5(IP) 0%, and 4(IP)/(6)IP 79%. Similar results were obtained in two other experiments.

Fig. 3. Identification of HPLC-purified [3H](1,3,4,5)IP4 from unstimulated and bombesin (BBS)-stimulated AR4-2J cells using 1,4,5)IP3/(1,3,4,5)IP4-5-phosphatase. HPLC-purified [3H]-labeled material (open symbols) from the middle inositol tetrakisphosphate peak from bombesin-stimulated (squares) or unstimulated (triangles) cells was combined with [32P](1,3,4,5)IP4 standard (closed symbols) and incubated with partially purified 5-phosphatase. At various times, the amounts of remaining [3H]IP, and [32P](1,3,4,5)IP4 standard were determined as described under "Experimental Procedures." These values are expressed as a percentage of the [3H]-labeled material and [32P]IP/(1,4,5)IP4 in each incubation before addition of the enzyme (time 0). Similar results were obtained in a second experiment. The difference in the rates of IP metabolism in incubations of material from stimulated versus unstimulated cells stems from the fact that similar amounts of radioactivity and, thus, a greater proportion of the HPLC-purified material from the unstimulated cell samples, were used in each assay. A component of the HPLC-purified material was slightly inhibitory for the 5-phosphatase, resulting in the relatively slower rate of metabolism for both the [3H]-labeled material and [32P]IP/(1,3,4,5)IP4, in the unstimulated cell incubations.

confirmed to be [3H](1,3,4,5)IP4 by the fact that the time course and extent of its metabolism by partially purified, stereospecific (21) (1,4,5)IP3/[1,3,4,5)IP4-5-phosphatase was distinguishable from that of authentic [32P]IP/(1,3,4,5)IP4 (Fig. 3).

The third eluting [3H]inositol tetrakisphosphate peak (Fig. 1) was believed to be one of the endoanionomic pair, (3,4,5,6)IP4/(1,4,5,6)IP4, based on its elution relative to [3H]IP4 (1,3,4,5)IP4, (6, 18; data not shown). These inositol tetrakisphosphates (but no others) yield iditol upon periodate oxidation, reduction, and dephosphorylation. When the [3H]-labeled material in this peak taken from unstimulated or bombesin stimulated cells was subjected to such a treatment, about 85% of the radioactivity was recovered as [3H]iditol, based on elution on the Polybore P column (three experiments, Fig. 4) and the Aminex column (one experiment, data not shown). The remaining radioactivity, eluting near inositol on the Polybore P column but clearly separated from inositol on the Aminex column (Fig. 4. insets), was not one of the specific polyols that could be directly derived from an inositol tetrakisphosphate. Thus, this material is believed to be a side product of the periodate procedure. (3,4,5,6)IP4 and (1,4,5,6)IP4 yield t-iditol and d-iditol, respectively; following oxidation, reduction, and dephosphorylation. A stereospecific polyol dehydrogenase was used to resolve the [3H]iditol enantiomers (see "Experimental Procedures") and thereby quantify the relative proportions of [3H]IP4/(1,3,4,5)IP4 and [3H]IP4/(1,3,4,5)IP4 in the third eluting inositol tetrakisphosphate peak. It was found that [3H]IP4 comprised 53 ± 14% (n = three experiments) of this peak in unstimulated cells and 90 ± 5% in stimulated cells. Thus, the AR4-2J cells appear to contain both (3,4,5,6)IP4 and (1,4,5,6)IP4. Furthermore, these data indicate that bombesin stimulation resulted primarily, if not exclusively, from progressive accumulation of the products of the quantitatively minor (1,4,5)IP4-3 kinase pathway, which were not detectable with the previously utilized protocols (15).
periodate oxidation, reduction, and dephosphorylation and the re-
panel) or bombesin-stimulated (lower panel) cells was subjected to
profile of standards (S'tds) of ["HI' mositol (Ins), [3H]altritol
besin-stimulated AR4-2J cells. The upperpanel shows the elution
incorporated into the [3H]inositol lipids and [3H]inositol
activity used, resulted in different levels of radioactivity being
72 h, 3 h, or pulse-chase with [3H]myo-inositol. These alter-
line hydrolysis (data not shown).

Inositol PO&phosphates--AR4-2J cells were labeled for either
coelution with [32P](IPs standard5 and by the fact that it
material from the cell samples did not coelute with ["Clglucitol (Glu), ["C]xylitol (Xyl), and [3H]iditol (Zdi) from a
Polybore Pb column eluted as described under "Experimental Pro-
cedures." HPLC-purified "H-labeled material (dotted line) from the
third inositol tetrakisphosphate peak from unstimulated (middle
panel) or bombesin-stimulated (lower panel) cells was subjected to
periodate oxidation, reduction, and dephosphorylation and the re-
sultant ["H-polys were separated using the Polybore Pb column.
Insets: The ["H-labeled material from the cells (dotted lines) that eluted with a retention time similar to the ["HI]inositol standard
(upper panel) was collected, lyophilized, and rechromatographed with
["C]inositol (solid lines) on an Aminex-H+ column. This ["H-labeled
material from the cell samples did not coelute with ["C]inositol on
this column (or on the Polybore Pb column, with any other polyol
that could be produced from an inositol tetrakisphosphate), suggesting
that it was a byproduct of the periodate procedure.

sively, in an increase in the (3,4,5,6)IP4 isomer.
The ["HI]inositol pentakisphosphate in the AR4-2J cells
was identified by alkaline hydrolysis as (1,3,4,5,6)IP5 (i.e. no
(2)IP was produced), and ["H]IP3 was identified based on its
coculation with [32P]IP3 standard5 and by the fact that it yielded
(1)IP(3)IP, (2)IP, (4)IP(6)IP, and (5)IP upon alkali-
line hydrolysis (data not shown).

Effect of Labeling Condition on ["HI]inositol Lipids and ["HI]
Inositol Polyphosphates—AR4-2J cells were labeled for either
72 h, 3 h, or pulse-chase with ["H]myo-inositol. These alter-
native labeling protocols, and the different amounts of radio-
activity used, resulted in different levels of radioactivity being incorporated into the ["HI]inositol lipids and ["HI]inositol
polyphosphates both prior to and during the experimental incu-

bations. It has been previously shown that incubation of AR4-
2J cells with ["H]myo-inositol for 72 h results in steady-state labeling of the ["HI]inositol lipids and, therefore, ["H]
(1,4,5)IP3 (13); 72 h of incubation with ["H]myo-inositol also results in steady-state labeling of ["H](3,4,5,6)IP4, but not
["H]IP3. Thus, the change in ["H]-labeled compounds during stimulation with bombesin in the 72-h group approximated changes in the mass of the inositol lipids and inositol phos-
phates (13). Furthermore, since the timing and duration of exposure to ["H]myo-inositol apparently does not affect total
inositol phosphate mass (see “Experimental Procedures”), it can be assumed that the differences across labeling groups in
the level of ["HI]inositol lipids and ["HI]inositol phosphates at any particular point during the experiments reflected differences in specific radioactivity.

At the start of the experimental incubations, the level of
radioactivity in the ["HI]inositol lipids in the 72-h group was higher than in the pulse-chase and 3 h groups (Fig. 5, upper
panel). In both the 72-h and pulse-chase groups, stimulation of the cells with bombesin caused a small but statistically
significant (p < 0.05) decrease in ["HI]inositol lipids level. In
contrast, bombesin stimulation of the 3-h group caused within
30 min a 4-fold increase in the radioactivity in the ["HI]inositol
lipids, reflecting a rapid increase in ["HI]inositol lipid-specific
radioactivity.

The amount of radioactivity in ["H](1,4,5)IP3 also varied considerably across labeling conditions in both stimulated
and unstimulated cells (Fig. 5, lower panel). As with the ["H]
inositol lipids, at the start of the experimental incubations the level of radioactivity in ["H](1,4,5)IP3 was highest in the
72-h group. Within 15 s of stimulation with bombesin, the
["H](1,4,5)IP3 levels increased approximately 10-fold in all
three groups. In the 72-h and pulse-chase groups, the ["H]
(1,4,5)IP3 level then decreased 50% from this peak and re-
mained elevated (Fig. 5, lower panel) for 4 h (data not shown).
However, in the 3-h group, the [3H](1,4,5)IP₃ level continued to increase for 30 min during stimulation, after which this elevated level of radioactivity was maintained.

Despite the differences in the levels and temporal patterns of change in radioactivity associated with the [3H]inositol lipids and [3H](1,4,5)IP₃, the ratio of [3H](1,4,5)IP₃ to the [3H]inositol lipids, expressed as % of the [3H]inositol lipid level, was very similar across labeling conditions in stimulated or unstimulated cells (Fig. 6, upper left panel). Since the specific radioactivities of [3H](1,4,5)IP₃ and the [3H]inositol lipids are the same in the 72-h group (13) and it is assumed that the (1,4,5)IP₃ mass relative to the inositol lipids is the same in each of the differently labeled groups (see "Experimental Procedures"), these data suggest that the specific radioactivities of the [3H]inositol lipids and [3H](1,5)IP₃ were very similar to one another in each group. This similarity was maintained even when the [3H]inositol lipid-specific radioactivity was rapidly increasing during bombesin stimulation in the 3-h group.

The levels of radioactivity in [3H](1,3,4)IP₃ and [3H] (1,3,4,5)IP₃ were near the limit of detection in unstimulated cells under all labeling conditions. Upon stimulation with bombesin, the levels of radioactivity in these [3H]inositol polyphosphates increased, and, as for [3H](1,4,5)IP₃, varied considerably in the differently labeled cells (data not shown). Nonetheless, the ratios of these [3H]inositol polyphosphates to the [3H]inositol lipids were very similar across labeling conditions (Fig 6). This suggests that these [3H]inositol polyphosphates had specific radioactivities similar to the [3H]inositol lipids and [3H](1,4,5)IP₃. The ratios of [3H](1,3,4)IP₃ and [3H](1,3,4,5)IP₃ to the [3H]inositol lipids increased in bombesin-stimulated cells in parallel and peaked after approximately 5 min. These elevated ratios thereafter remained essentially constant through 4 h of stimulation.

The first eluting [3H]inositol tetrakisphosphate peak (Fig. 1) was comprised of [3H](1,3,4,6)IP₄, and a second [3H]inositol tetrakisphosphate isomer. The ratio of the total radioactivity in this peak to that in the [3H]inositol lipids in unstimulated cells was approximately 2-fold higher in the pulse-chase group, and 2-fold lower in the 3-h group, compared with the 72 h group (Fig. 6, bottom right panel). This variation across labeling conditions contrasted with the data for [3H](1,4,5)IP₃ and indicated that in unstimulated cells, the turnover of one or both of the [3H]inositol tetrakisphosphate isomers comprising the first eluting peak was slower than that of the [3H]inositol lipids. Stimulation of the cells with bombesin increased the radioactivity in the first eluting [3H]inositol tetrakisphosphate peak in all groups (data not shown), predominantly due to an increase in [3H](1,3,4,6)IP₄ (see above). Bombesin stimulation also decreased the difference across labeling conditions in the ratio of radioactivity in the this peak to that in the [3H]inositol lipids (Fig. 6, bottom right panel). This suggested that in the stimulated cells [3H] (1,3,4,6)IP₄ had a specific radioactivity similar to that of the [3H]inositol lipids.

The several [3H]inositol polyphosphates discussed above that appear to have specific radioactivities similar to the [3H]inositol lipids (i.e. (1,4,5)IP₃, (1,3,4)IP₃, (1,3,4,5)IP₃, and (1,3,4,6)IP₄) contrast with a second group of [3H]inositol polyphosphates, [3H](1,3,4,5)IP₄, [3H](1,4,5,6)IP₄, and [3H] (3,4,5,6)IP₄; the ratio of radioactivity in both [3H] (1,3,4,5,6)IP₄ (Fig. 7, lower panel) and the [3H](1,4,5,6)IP₄/...
Inositol Tetrakisphosphates in AR4-2J Cells

It is becoming increasingly clear that the synthesis and metabolism of the inositol polyphosphates is a complex process that occurs in organisms across the phylogenetic spectrum.

**DISCUSSION**

Radioactivity in [3H]IP₆ was detected in the pulse-chase and 72-h groups but not in the 3-h group (data not shown). The ratio of [3H]IP₆ to [3H](1,3,4,5,6)IP₆ was higher in the pulse-chase group than in the 72-h group (Fig. 9); thus, the turnover of [3H]IP₆ appeared slower than that of the other [3H]inositol phosphates considered above. Incorporation of radioactivity into IP₆ was not affected by bombesin.

In all labeling groups, bombesin stimulation increased the level of radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak (Fig. 8, upper panel), primarily due to an increase in [3H](3,4,5,6)IP₄ (see above). Importantly, the ratio of radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak to that in [3H](1,3,4,5,6)IP₅ was very similar across labeling groups in stimulated cells (Fig. 8, lower panel). This suggested that the specific radioactivities of [3H](3,4,5,6)IP₅ and [3H](1,3,4,5,6)IP₅ were very similar among all labeling groups. In resting cells, radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak was reliably detected in the 72-h and pulse-chase groups (Fig. 8, upper panel). In these groups, the ratios of radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak to that in [3H](1,3,4,5,6)IP₅ were very similar (Fig. 8, lower panel). Since the proportion of [3H]IP₆ to [3H](1,4,5,6)IP₅ to [3H](1,3,4,5,6)IP₅ was highest in the resting cells, these data suggested that the specific radioactivity of [3H](1,4,5,6)IP₅ was also similar to that of [3H](1,3,4,5,6)IP₅.

Radioactivity in [3H]IP₆ was detected in the pulse-chase and 72-h groups but not in the 3-h group (data not shown). The ratio of [3H]IP₆ to [3H](1,3,4,5,6)IP₅ was higher in the pulse-chase group than in the 72-h group (Fig. 9); thus, the turnover of [3H]IP₆ appeared slower than that of the other [3H]inositol phosphates considered above. Incorporation of radioactivity into IP₆ was not affected by bombesin.

**FIG. 8.** The effect of labeling condition on [3H](1,3,4,5,6)IP₅ in unstimulated and bombesin (BBS)-stimulated AR4-2J cells. Cells were labeled for 3 h (dashed line), 72 h (solid line), or pulse-chase (dotted line) with [3H]myo-inositol as described under "Experimental Procedures." The cells were then incubated in the absence (open symbols) or presence (closed symbols) of 200 nM bombesin for up to 240 min and [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄ was determined. The upper panel depicts the level of [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄ (referred to as [3H](3,4,5,6)IP₄ on the ordinate) was determined. The upper panel depicts the level of [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄ in dpm/well. In the middle panel, the level of [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄ is expressed as a percentage of the level of [3H]inositol lipids (%IL). For the lower panel, the level of [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄ is expressed as a percentage of the level of [3H](1,3,4,5,6)IP₅ (%IP₅). Error bars are smaller than the symbols when not shown.

**FIG. 7.** The effect of labeling condition on [3H](1,3,4,5,6)IP₅ in unstimulated and bombesin (BBS)-stimulated AR4-2J cells. AR4-2J cells were labeled for 3 h (dashed line), 72 h (solid line), or pulse-chase (dotted line) with [3H]myo-inositol as described under "Experimental Procedures." The cells were then incubated in the absence (open symbols) or presence (closed symbols) of 200 nM bombesin for up to 240 min and [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄ was determined. The upper panel depicts the level of [3H](3,4,5,6)IP₄/[3H](1,4,5,6)IP₄, in dpdm/well as a function of time. For the lower panel, the level of [3H](1,4,5,6)IP₄ in each well was converted to a percentage of the level of [3H]inositol lipids in that well (%IL). Error bars are smaller than the symbols when not shown.

[3H](3,4,5,6)IP₄, peak (Fig. 8, middle panel, referred to as DL(3,4,5,6)IP₄) to that in the [3H]inositol lipids were 3-fold higher in the pulse-chase group, and 25-fold or more lower in the 3-h group, compared with the 72-h group. These data suggest that the turnovers of [3H](1,3,4,5,6)IP₅, [3H](1,4,5,6)IP₄, and/or [3H](3,4,5,6)IP₄ were slower than that of the [3H]inositol lipids.

The level of radioactivity in [3H](1,3,4,5,6)IP₅ was substantially higher than that of the other [3H]inositol polyphosphates in the 72-h group (see Fig. 1), indicating that it was of the greatest mass (with the possible exception of IP₅ which was not labeled to steady state). Agonist stimulation altered accumulation of radioactivity (approximately 950 dpm/h/well) in [3H](1,4,5,6)IP₄ in unstimulated cells. Bombesin increased the rate of [3H](1,3,4,5,6)IP₅ accumulation over 3-fold (to approximately 3200 dpm/h/well). In contrast to the 3-h group, in both the 72-h and pulse-chase group, the [3H](1,3,4,5,6)IP₅ level remained relatively constant in unstimulated cells. Moreover, in six separate experiments in cells labeled for 72 h, the [3H](1,3,4,5,6)IP₅ level after 60 min of stimulation decreased 7.8 ± 1.3% (p ≤ 0.05); the magnitude of the decrease was similar in the pulse-chase group (n = three experiments, p ≤ 0.05).

In all labeling groups, bombesin stimulation increased the level of radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak (Fig. 8, upper panel), primarily due to an increase in [3H](3,4,5,6)IP₄ (see above). Importantly, the ratio of radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak to that in [3H](1,3,4,5,6)IP₅ was very similar across labeling groups in stimulated cells (Fig. 8, lower panel). This suggested that the specific radioactivities of [3H](3,4,5,6)IP₅ and [3H](1,3,4,5,6)IP₅ were very similar within all labeling groups. In resting cells, radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak was reliably detected in the 72-h and pulse-chase groups (Fig. 8, upper panel). In these groups, the ratios of radioactivity in the [3H](1,4,5,6)IP₄/[3H](3,4,5,6)IP₄ peak to that in [3H](1,3,4,5,6)IP₅ were very similar (Fig. 8, lower panel). Since the proportion of [3H]IP₆ to [3H](1,4,5,6)IP₅ to [3H](1,3,4,5,6)IP₅ was highest in the resting cells, these data suggested that the specific radioactivity of [3H](1,4,5,6)IP₅ was also similar to that of [3H](1,3,4,5,6)IP₅.
The current interest in this process in mammalian tissues owes much to the following two discoveries: 1) that (1,4,5)IP3, the first product of receptor-activated breakdown of PIP2, is the intracellular messenger which initiates the process of Ca2+ mobilization (2), and 2) that (1,4,5)IP3 is but one of a number of inositol polyphosphates that increase during receptor activation (1). Some of which appear to have biological actions of differing relative specific radioactivities for candidates (1,3,4,6)IP4 and (3,4,5,6)IP4. Both of these inositol tetrakisphosphates have been shown to be phosphorylated to (1,3,4,6)IP5 in vitro (7, 11, 18, 27, 28). The differential effects of labeling condition on the ratios of radioactivity in (1,3,4,6)IP4 to (1,3,4,5,6)IP5 in intact AR4-2F cells suggest that (1,3,4,6)IP4 is the major, if not exclusive, precursor for de novo (1,3,4,5,6)IP5 synthesis. The arguments that support this suggestion are as follows. First, comparison of the ratios of (1,3,4,6)IP4 and (1,3,4,5,6)IP5 to the [3H]inositol lipids in unstimulated cells of the 3-h and 72-h groups suggest that, in the 3-h group, the specific radioactivity of the (1,3,4,6)IP4 was substantially greater than that of (1,3,4,5,6)IP5 and for the [3H]inositol lipids in unstimulated cells of the 3-h and 72-h groups. Second, in the 72-h group, where [3H]inositol lipids were labeled to isotopic steady state, a small decrease in (1,3,4,5,6)IP5 was stimulated by bombesin. A similar decrease was observed in the pulse-chase group, where comparison of the ratios of (1,3,4,6)IP4 and [3H]inositol lipids in unstimulated cells suggests that the specific radioactivity of [3H](1,3,4,5,6)IP5 was higher than that of (1,3,4,6)IP4. Thus, accumulation of radioactivity in [3H](1,3,4,5,6)IP5 is observed. Stimulation of the cells in this group with bombesin caused an apparent 4-fold increase in the specific radioactivity of the [3H]inositol lipids and [3H](1,4,5)IP3 and, therefore, an increase of at least this magnitude in the specific radioactivity of [3H](1,3,4,5,6)IP5. This was accompanied by a greater than 3-fold increase in the rate of incorporation of radioactivity into [3H](1,3,4,6)IP4. Second, in the 72-h group, where [3H](1,3,4,5,6)IP4 was labeled to isotopic steady state, a small decrease in (1,3,4,5,6)IP5 was stimulated by bombesin. A similar decrease was observed in the pulse-chase group, where comparison of the ratios of (1,3,4,6)IP4 and [3H]inositol lipids in unstimulated cells suggests that the specific radioactivity of [3H](1,3,4,5,6)IP5 was higher than that of (1,3,4,6)IP4. Thus, accumulation of radioactivity in [3H](1,3,4,5,6)IP5 is observed only under the labeling condition (3 h) where the specific radioactivity of [3H](1,3,4,5,6)IP5 is thought to be substantially higher than that of [3H](1,3,4,5,6)IP4. Furthermore, the bombesin-stimulated increase in accumulation of [3H](1,3,4,5,6)IP5 in the 3-h group can be accounted for by the apparent increase in (1,3,4,6)IP4-specific radioactivity. In contrast, the phosphorylation of [3H](3,4,5,6)IP4 could not account for these differential effects of labeling condition on the incorporation of radioactivity into [3H](1,3,4,5,6)IP5; since the ratio of radioactivity in [3H](3,4,5,6)IP, to [3H](1,3,4,5,6)IP5, and therefore, presumably their specific radioactivities, were similar under all the labeling conditions. Thus, (1,3,4,6)IP4, rather than (3,4,5,6)IP4, is suggested to be the precursor for the de novo synthesis of (1,3,4,5,6)IP5.

As pointed out by Carpenter et al. (30), the regulation of
synthesis and metabolism of IPs appears to vary with cell type. For example, IP₃ has been reported to decrease (31), or remain unchanged (23, 30, 32) in different tissues during the activation of phospholipase C. In angiotensin II-stimulated adrenal glomerulosa cells, Balla et al. (6) reported an initial decrease followed by an increase in IP₃ level. In the AR4-2J cells, agonist activation appears to result in a net breakdown of (1,3,4,5,6)IP₃, as indicated by the small decrease induced by bombesin in [³H](1,3,4,5,6)IP₃ level under the condition (72 h) where [³H](1,3,4,5,6)IP₃ is labeled to steady state. Furthermore, in the 3-h group, the agonist-stimulated increase in [³H](1,3,4,5,6)IP₃ apparent specific radioactivity is of similar magnitude to the agonist-stimulated increase in rate of accumulation of [³H](1,3,4,5,6)IP₃ (see above) suggesting that activation of phospholipase C does not result in increased (1,3,4,5,6)IP₃ synthesis. (3,4,5,6)IP₄ appears to be the product of agonist-activated (1,3,4,5,6)IP₃ breakdown. This is suggested by the finding that [³H](1,4,5,6)IP₃, was increased in bombesin-stimulated cells while retaining the same specific radioactivity as [³H](1,3,4,5,6)IP₃ in each of the differently labeled groups. No [³H]inositol phosphate was detected other than [³H](1,3,4,5,6)IP₃, which was of an equivalent specific radioactivity and of sufficient mass to account for this increase in [³H](3,4,5,6)IP₃ during stimulation. The present results, however, do not rule out the possibility that the phosphorylation of (3,4,5,6)IP₃ to (1,3,4,5,6)IP₃ occurs as part of a futile cycle for the interconversion of these two inositol polyphosphates in the AR4-2J cells. If such were the case, then agonist activation could preferentially either stimulate the rate of (1,3,4,5,6)IP₃ dephosphorylation or inhibit the rate of (3,4,5,6)IP₃ phosphorylation. In either case, the increase in [³H](3,4,5,6)IP₄ level derives from a net loss of [³H](1,3,4,5,6)IP₃.

[³H](1,4,5,6)IP₃, was also detected in the AR4-2J cells; this isomer has not previously been detected in any cell type. Studies of broken cell preparations have shown that (1,4,5,6)IP₃ can be formed by (1,3,4,5,6)IP₃ dephosphorylation (18). In intact AR4-2J cells, the specific radioactivity of [³H](1,4,5,6)IP₃ was apparently similar to that of [³H](1,3,4,5,6)IP₃, consistent with the hypothesis that (1,4,5,6)IP₃ is a (1,3,4,5,6)IP₃ metabolite. However, the level of [³H](1,4,5,6)IP₃ did not appear to increase during agonist activation.

A fifth inositol tetrakisphosphate was also detected in the AR4-2J cells which coeluted with [³H](1,3,4,6)IP₄. This was identified as either (1,2,3,4,6)IP₄ or (1,2,3,5,6)IP₄. This is the first report of an inositol tetrakisphosphate isomer containing a 2-phosphate in any cell type. IP₆ was also detected in AR4-2J cells; the present results indicate that its turnover was slower than both the inositol lipids and (1,3,4,5,6)IP₃, although the metabolic pathways in the life cycle of IP₆ remain to be identified. Nonetheless, it is tempting to speculate that the metabolism of this fifth inositol tetrakisphosphate may be related to that of IP₆ since these were the only inositol polyphosphates detected which contained a 2-phosphate.

The results of the present study add the AR4-2J cells to the growing list of mammalian and non-mammalian cells in which multiple inositol tetrakisphosphates and higher inositol polyphosphates have been detected. More importantly, these results give the first clue as to the metabolic origin of (3,4,5,6)IP₃, in a mammalian cell and suggest the existence of an as yet unidentified (1,3,4,5,6)IP₃, 1-phosphatase which may be under hormonal control. We note that since the level of (1,3,4,5,6)IP₃ decreased slightly during bombesin stimulation of the AR4-2J cells, the activation of (3,4,5,6)IP₃ formation from (1,3,4,5,6)IP₃ must result from a regulated change in one or more of the intrinsic kinetic properties for the enzyme(s) involved in this pathway. Preliminary results (not data shown) indicate that the breakdown of [³H](3,4,5,6)IP₃, to [³H](3,4,5,6)IP₃, in the AR4-2J cells is not stimulated by activating protein kinase C with phorbol esters or by increasing the intracellular Ca²⁺ concentration with the tumor promoter thapsigargin (which inhibits certain intracellular Ca²⁺-ATPases and, thus, mimics the effect of (1,4,5)IP₃ on intracellular Ca²⁺ levels, (29)) or by both manipulations combined. At present the functions of (3,4,5,6)IP₄, and (1,3,4,5,6)IP₃ are uncertain. Vallejo et al. (22) have suggested that (3,4,5,6)IP₃, and (1,3,4,5,6)IP₃, may act as extracellular signaling molecules, at least in neurons. However, we note in AR4-2J cells, as in other cell types where it has been detected, that although the mass of IP₃ is relatively high, its level during agonist activation is affected to a much lesser degree than that of the less highly phosphorylated inositol phosphates, including (3,4,5,6)IP₃. On the other hand, it is more generally found that agonists stimulate increases in (3,4,5,6)IP₃, (7, 18, 33 and the present study). Thus, attention may be profitably focused on (3,4,5,6)IP₃ in a likely candidate for a modifier of intracellular regulatory processes in response to receptor activation. In this regard, Hughes et al. (32) have reported that (3,4,5,6)IP₃, is a potent inhibitor of the (1,3,4)IP₃, 6-kinase in vitro, suggesting that (3,4,5,6)IP₃, may function in the physiological regulation of this enzyme. Furthermore, since the formation of (3,4,5,6)IP₃, is apparently regulated at the level of the enzymes which interconvert (3,4,5,6)IP₃, and (1,3,4,5,6)IP₃, but not by a change in (1,3,4,5,6)IP₃, concentration, it is possible that this pathway may also be regulated independently of phospholipase C activation. Thus, further study is required, perhaps using the labeling techniques developed in the present study, to determine whether (1,3,4,5,6)IP₃, gives rise to (3,4,5,6)IP₃, in other cell types, to characterize the putative (1,3,4,5,6)IP₃, 1-phosphatase, and to determine the mechanism(s) for the intracellular regulation of this pathway.

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REFERENCES
Inositol Tetrakisphosphates in AR4-2J Cells

Origins of myo-inositol tetrakisphosphates in agonist-stimulated rat pancreatoma cells. Stimulation by bombesin of myo-inositol 1,3,4,5,6-pentakisphosphate breakdown to myo-inositol 3,4,5,6-tetrakisphosphate.

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