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FORMATION OF INOSITOL PENTAKISPHOSPHATE BY OVARIAN FOLLICLES OF *XENOPUS LAEVIS* FROM METABOLISM OF INOSITOL (1,4,5)TRISPHOSPHATE AND INOSITOL (1,3,4,5)TETRAKISPHOSPHATE AND FROM RECEPTOR ACTIVATION

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SUMMARY: Small amounts of a higher inositol phosphate with chromatographic properties of [^{3}H]inositol (1,3,4,5,6)pentakisphosphate were formed from [^{3}H]inositol (1,4,5)trisphosphate added to homogenates of ovarian follicles of *Xenopus laevis*, and from [^{3}H]inositol (1,3,4,5)tetrakisphosphate after injection into follicular oocytes. Other intermediate forms of inositol tetrakisphosphate were not detectable. [^{3}H]inositol (1,3,4,5,6)pentakisphosphate prepared from chicken erythrocytes was metabolized in homogenates to an inositol tetrakisphosphate eluting later than the (1,3,4,5) isomer. Activation of receptors in ovarian follicles of *Xenopus laevis* with acetylcholine or stimulation with injected GTPγS caused formation not only of inositol trisphosphate and its expected metabolites but also of small amounts of inositol pentakisphosphate. These results suggest that the latter may be formed from metabolites of inositol (1,4,5)trisphosphate in this tissue during receptor activation.

In many cell types, agonist binding to specific receptors has been shown to cause activation of the enzyme phospholipase C and hydrolysis of phosphatidyl Ins(4,5)P_2. The products formed, 1,2-diacylglycerol and Ins(1,4,5)P_3, both act as intracellular signals, the former by activating protein kinase C and the latter by releasing intracellular Ca^{2+}. The subsequent metabolism of Ins(1,4,5)P_3 and its products by kinases and phosphatases is being revealed as increasingly complex [reviewed, for example, in (1)], with at least some other metabolites appearing to have physiological activity [e.g. Ins(1,3,4,5)P_4 modulation of cellular Ca^{2+} fluxes (2, 3)]. An elegant recent study (4) has demonstrated the ability of homogenates of rat brain and of mouse macrophages to form Ins(1,3,4,5,6)P_5 from Ins(1,4,5)P_3, and homogenates of rat brain to form Ins(1,3,4,5,6)P_5 from Ins(1,3,4,5)P_4. Intact macrophages were shown to produce small amounts of InsP_5 phosphate during agonist stimulation. This suggests that

Abbreviations: GTPγS, guanosine 5'-O-(3-thiotriphosphate); Ins, *myo*-inositol; InsP_2, *myo*-inositol bisphosphate; InsP_3, *myo*-inositol trisphosphate; InsP_4, *myo*-inositol tetrakisphosphate; InsP_5, *myo*-inositol pentakisphosphate (with isomeric numbering as appropriate; all D-enantiomers).

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receptor activation may play a part in producing Ins(1,3,4,5,6)P_5 in addition to previous reports of its production from Ins(3,4,5,6)P_4 independently of the action of agonists (5).

Evidence is presented here that ovarian follicles of *Xenopus laevis* can not only produce an InsP_5 (with the chromatographic properties of Ins(1,3,4,5,6)P_5) upon stimulation with acetylcholine and by microinjection of the G-protein activator GTPγS, but also that InsP_5 can be formed by intact cells from the injected transduction product Ins(1,3,4,5)P_4 and by homogenates from Ins(1,4,5)P_3. These results mutually reinforce the probability that receptor activation of *Xenopus* ovarian follicles can produce InsP_5.

MATERIALS AND METHODS

Animals

Mature female *Xenopus laevis* were obtained from NASCO (Fort Atkinson, Wisconsin) or from the Universities of Christchurch or Auckland, New Zealand. They were fed minced beef liver twice a week and maintained at about 20 °C in dechlorinated tap water containing 50 mg EDTA/l, with a 14/10 h light/dark cycle. They were anaesthetized with 3-aminobenzoic acid ethyl ester methane sulphonate salt (0.2% w/v) or on ice during surgical removal of ovarian tissue.

Materials and HPLC

[^{3}H]Labelled Ins (61.2 Ci/mmol), Ins(1,4)P_2 (1.5 Ci/mmol), Ins(1,4,5)P_3 (4 Ci/mmol), and Ins(1,3,4,5)P_4 (0.1 and 17 Ci/mmol) were obtained from Dupont/NEN Products. [^{3}H]Ins(1,3,4)P_3 was formed from [^{3}H]Ins(1,3,4,5)P_4 using red blood cell membrane 5'-phosphomonoesterase (6), and [^{3}H]Ins(1,3,4,5,6)P_5 was isolated from chicken erythrocytes labelled with [^{3}H]Ins (4). [^{3}H]Ins phosphates were purified by HPLC, eluted from Dowex-8 with ammonium formate, and salt was removed by lyophilization in polypropylene tubes (to avoid losses of the higher phosphoinositols) before uptake in water. [^{3}H]Ins was purified by HPLC and lyophilization immediately before use.

Samples of ~ 20 ovarian follicles at stages 5 and 6 were dissected from ovarian tissue in OR-2 medium. The follicles were either labelled with [^{3}H]Ins and stimulated with hormone, or [^{3}H]Ins phosphates were injected or added to follicular homogenates. Follicles were stimulated with acetylcholine (200 μM) after incubation in 100 μCi [^{3}H]Ins/ml overnight or for two days, followed by thorough washing. LiCl (10 mM) was added 5 min before hormone addition. Samples of 20 follicles were homogenized by 0.5 s sonication with a microprobe in 55 μl of a solution containing 250 mM sucrose, 50 mM Tris/HCl, pH 7.3, 1 mM EGTA, 1 μg leupeptin/ml, 10 mg bovine serum albumin/ml, and 50 μM phenylmethane-sulphonyl fluoride. The volume was made up to 200 μl to give final concentrations of 50 mM potassium glutamate, 28 mM NaCl, 1 mM EGTA, 4 mM $MgSO_4$, 5 mM ATP, and 7 mM HEPES, pH 7.2 (7). About 15 nCi of [^{3}H]InsP was added to homogenates. Ins(1,3,4)P_3 (0.5 nCi, about 0.3 fmol), Ins(1,4,5)P_3 (0.5 nCi, 0.13 fmol), Ins(1,3,4,5)P_4 (0.5 nCi, 0.3 fmol or 5 fmol) or GTPγS (Sigma; 35 pmol) were microinjected in 50 nl into ovarian follicles. These solutions were taken up into a glass needle (aperture 20 μm) filled with paraffin oil and attached to a Hamilton Microlab-P programmable pipette set to deliver 50 nl. Reactions were terminated and Ins phosphates extracted as described by (8), but with phytic acid hydrolysate (35 μg P_i/sample) added to the extraction medium to minimize loss of Ins(1,4,5)P_3 (9).

Ins phosphates were analysed by HPLC (10) using a Perkin Elmer Series 410 BIO pump and controller, and 250 x 4.6 mm SAX columns (Alltech or Hichrom) with linear or concave gradients from 0–1.6 M ammonium phosphate, pH 3.35 (79.3 g diammonium hydrogen phosphate/l plus about 60 ml orthophosphoric acid) over 70–90 min designed to optimize resolution of peaks of interest. Eluent (1 ml/min) was mixed continuously with ACS II scintillant (Amersham International) and counted using a Nuclear Enterprises Isoflo 1 flow liquid scintillation detector, or

collected in 0.5 ml fractions for prolonged counting in a Beckman liquid scintillation spectrometer. Counts from the Isoflo 1 were summed over 6 s intervals using a serializing interface and Macintosh microcomputer that displayed results in real time and stored the data for later analysis. Peaks were identified by comparison of their elution volumes with standards ([^{3}H]Ins, [^{3}H]Ins(1,4)P_2, [^{3}H](1,3,4)P_3, [^{3}H](1,4,5)P_3, [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,3,4,5,6)P_5) run under identical conditions about every fourth sample. Results from compared samples were normalized for total counts and usually smoothed (11) before presentation. All experiments were repeated 3–5 times with qualitatively the same results.

RESULTS AND DISCUSSION

Metabolism of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5

On both addition of Ins(1,4,5)P_3 to homogenates of ovarian follicles and after injection of Ins(1,4,5)P_3 into follicles, Ins(1,3,4,5)P_4 and InsP_2 were formed rapidly, and Ins(1,3,4)P_3, InsP_1 and inositol more slowly. Similar metabolism of this substance has been observed in many other tissues from other species (e.g. 12–15). In addition, however, a small peak was seen eluting at the position of Ins(1,3,4,5,6)P_5 after metabolism of Ins(1,4,5)P_3 in homogenates of ovarian follicles for 25 min or more (Fig. 1*c*; n = 7). Although the count rates were low, fractions were collected by hand and counted sufficiently long for this peak to be replicated unequivocally in comparison with baseline counts. Other InsP_4 peaks formed in addition to Ins(1,3,4,5)P_4 which have been proposed as intermediates in forming the higher phosphate (e.g. 16, 14, 4, 5) were not evident.

A peak eluting at the position of Ins(1,3,4,5,6)P_5 was also readily seen in oocytes injected with Ins(1,3,4,5)P_4 (n = 12; Fig 2*b*), but was not evident in oocyte homogenates (n = 14; Fig 2*a*). Oocytes caused to mature by treatment with 1 μM

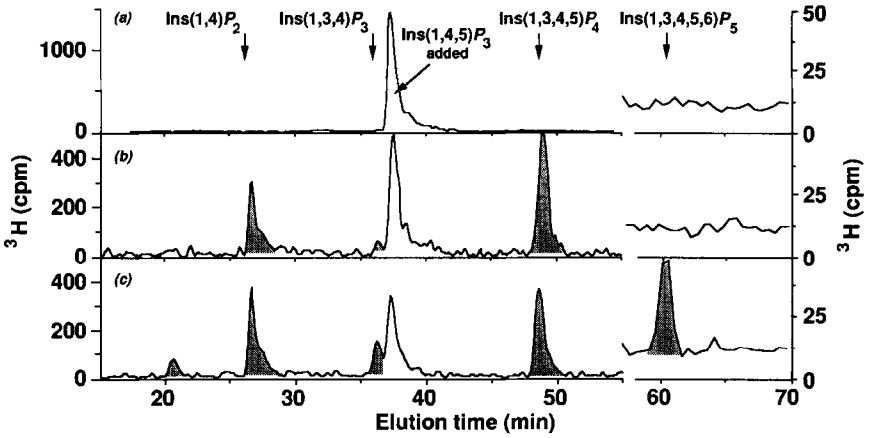

Figure 1. **Metabolism of Ins(1,4,5)P_3 in homogenates of ovarian follicles.** Samples were extracted (*a*) 0, (*b*) 8, or (*c*) 25 min after addition of [^{3}H]Ins(1,4,5)P_3 to reveal the time course of its metabolism. Results on the auxiliary scales to the right were obtained from samples collected by a fractionator and counted for longer times, using standard liquid-scintillation methods. *Shading* indicates the formation of metabolic products. Elution times of standards are shown by *arrows*.

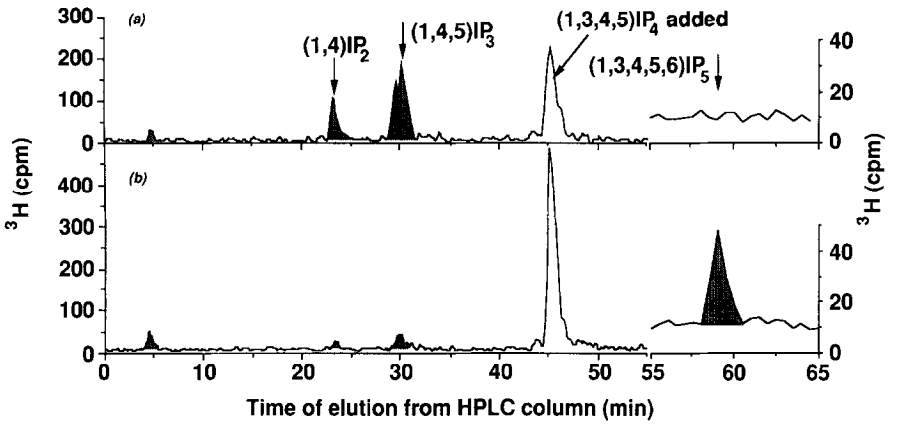

Figure 2. **Metabolism of Ins(1,3,4,5)P_4 in homogenates of ovarian follicles, and injected into whole follicles.** Samples were extracted 30 min after addition of [^{3}H]Ins(1,3,4,5)P_4 (0.1 Ci/mmol) to (*a*) homogenates or (*b*) after injection of tracer into whole follicles. Results shown on the auxiliary scales to the right were obtained from samples collected by a fractionator and counted for longer times using standard liquid-scintillation methods. *Shading* indicates the formation of metabolic products. Elution times of standards are shown by *arrows*.

progesterone formed larger amounts of InsP_5 (10%) from more rapid metabolism (70%) of injected Ins(1,3,4,5)P_4 (17 Ci/mmol) during 20 min.

Follicle homogenates metabolized Ins(1,3,4,5,6)P_5 over 45 min to an InsP_4 which was not Ins(1,3,4,5)P_4 (Fig. 4, n = 3) but which was eluted at higher concentrations of salt. In homogenates of other tissues this metabolic product of Ins(1,3,4,5,6)P_5 has been identified as Ins(1,4,5,6)P_4 (5, 17).

Evidence has been presented that the Ins(1,3,4,5)P_4 metabolite Ins(1,3,4)P_3 is further phosphorylated to another isomer of InsP_4, Ins(1,3,4,6)P_4 in permeabilized bovine adrenal glomerolosa cells and rat liver homogenates (15, 13), and this isomer as well as Ins(3,4,5,6)P_4 can be phosphorylated to InsP_5 (5, 16). These isomers of InsP_4 were absent or below detection levels during production of InsP_5 in whole or homogenized follicles. While Ins(3,4,5,6)P_4 as a stereoisomer of Ins(1,4,5,6)P_4 would be expected to be distinguished chromatographically from Ins(1,3,4,5)P_4 (Fig. 4) if present in measurable amounts, it was possible that Ins(1,3,4,6)P_4 was formed but not resolved, or that because these isomers may have been slow to form but were phosphorylated rapidly, peaks were not detected in our experiments.

Formation of InsP_5 from acetylcholine and GTPγS stimulation

Three out of five batches of follicles in which InsP_5 was looked for after stimulation with acetylcholine, showed a detectable increase in radioactivity at the elution time expected for this metabolite [n = 4; for example, Fig. 3, where the peak with the elution time of Ins(1,3,4,5,6)P_5 extracted from chicken red blood cells contained 13% of the counts in the InsP_3 peak formed during 60 min stimulation of follicles with 200 μM acetyl choline in 10 mM Li^+]. The peak eluting later at about 45 min was not identified, but may have been InsP_6. Injection of 35 pmol GTPγS into

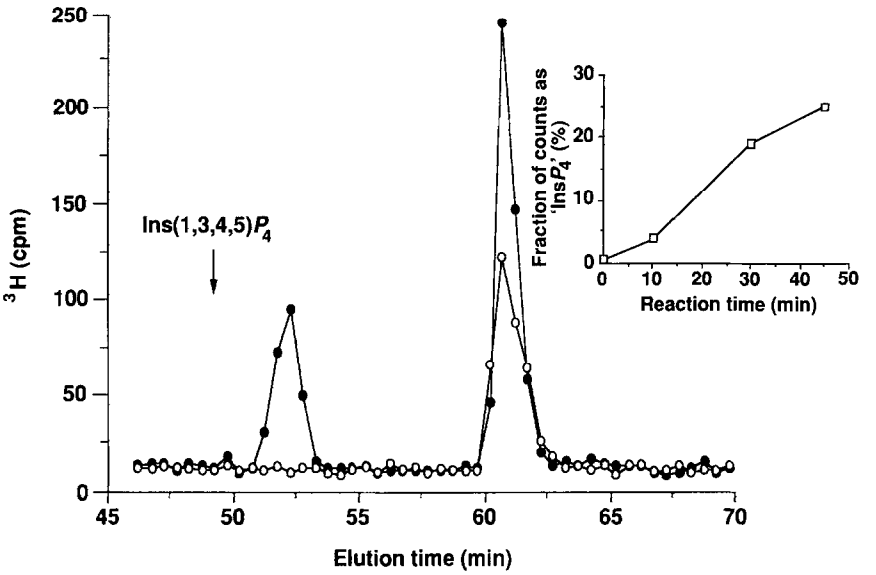

Figure 3. **Metabolism of [^{3}H]Ins(1,3,4,5,6)P_5 in ovarian follicle homogenates.** Samples were extracted (o) 0, or (•) 45 min after addition of tracer to homogenates and subjected to HPLC. No other metabolites were detected. The *insert* shows the rate of formation of an InsP_4 as a percentage of total counts added. A metabolite of InsP_5, formed under comparable conditions in other tissues and with similar chromatographic properties, has been identified as Ins(1,4,5,6)P_4 (5, 16).

follicles, in addition to increasing levels of InsP_1, InsP_2 and InsP_3 above controls, also formed apparent InsP_5 (to a level of 7% of the InsP_3 peak formed during 45 min incubation in 10 mM Li^+; n = 3). This peak was not formed by control follicles injected with water (n = 2). Different batches of follicles vary in many properties, including endogenous responsiveness to hormones and the unstimulated levels of

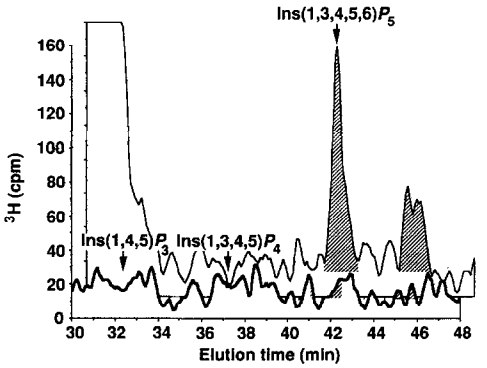

Figure 4. **Formation of apparent pentakis- and hexakisphosphates on stimulation of ovarian follicles with acetylcholine.** Follicles were incubated with [^{3}H]Ins (100 μCi/ml) for two days, washed thoroughly, and (—) stimulated with 200 μM acetylcholine for 60 min in 10 mM Li^+ before extraction or (—) left unstimulated as controls. Elution times of standards measured by HPLC in consecutive samples are shown by the *arrows*. The peak eluting at longest time from the stimulated sample was not characterized but is likely to be InsP_6.

baseline inositol phosphates formed on loading or injecting them with freshly-purified [^{3}H]Ins [e.g. (8)]. Variation between batches of follicles in amounts of enzymes forming InsP_5 is presumably related to season and maturity of follicles which can be only partially controlled in animals maintained in the laboratory.

Small or insignificant changes have been shown acutely in production of these higher inositol phosphates in other cell types [e.g. (18), over 5 min; (19), over 10 min; (20), over 40 min; (21), over 1 min; (5), over 10 min]. Proposed possible functions of inositol pentakisphosphate production, such as to act at receptors for Ins(1,3,4,5)P_4 and to mobilize cellular Ca^{2+} (22) and as longer term storage of phosphate needed during secretion, are speculative at present (23). InsP_5 and InsP_6 have also been proposed to have extracellular function in brain (24) and is believed to regulate the affinity of hemoglobin for oxygen in avian erythrocytes (25). Receptor stimulation of InsP_5 formation in ovarian follicles would appear to be longer term, and an apparently minor pathway in the overall metabolism of receptor-stimulated inositol phosphates.

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