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FORMATION OF INOSITOL 1,3,4,6-TETRAKISPHOSPHATE DURING ANGIOTENSIN II ACTION IN BOVINE ADRENAL GLOMERULOSA CELLS

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Summary: Angiotensin II stimulates the formation of several inositol polyphosphates in cultured bovine adrenal glomerulosa cells prelabelled with [^{3}H]inositol. Analysis by high performance anion exchange chromatography of the inositol-phosphate compounds revealed the existence of two additional inositol tetrakisphosphate ($InsP_4$) isomers in proximity to Ins-1,3,4,5-P_4, the known phosphorylation product of Ins-1,4,5-trisphosphate and precursor of Ins-1,3,4-trisphosphate. Both of these new compounds showed a slow increase after stimulation with angiotensin II. The structure of one of these new $InsP_4$ isomers, which is a phosphorylation product of Ins-1,3,4-P_3, was deduced by its resistance to periodate oxidation to be Ins-1,3,4,6-P_4. The existence of multiple cycles of phosphorylation-dephosphorylation reactions for the processing of Ins-1,4,5-P_4 may represent a new aspect of the inositol-lipid related signalling mechanism in agonist-activated target cells.

Angiotensin II exerts its steroidogenic effect on the adrenal glomerulosa zone through Ca^{2+}-mobilizing receptors in the plasma membrane (1-3). Binding of angiotensin II to such receptors results in rapid breakdown of PtdIns-4,5-P_2 (4,5) to yield Ins-1,4,5-P_3 (6) and DAG (7), both of which serve a second messenger role in various tissues (8,9). The active water-soluble species, Ins-1,4,5-P_3, has been shown to release Ca^{2+} from a non-mitochondrial pool (10) by a mechanism involving binding of Ins-1,4,5-P_3 to an intracellular receptor (11-13). Elimination of the Ins-1,4,5-P_3 signal seems to be extremely rapid and occurs mainly by an active dephosphorylating mechanism (14). The discovery of another pathway by which Ins-1,4,5-P_3 is converted to Ins-1,3,4-P_3 through Ins-

The abbreviations used are: Ins: inositol; Ins-1,4,5-P_3: inositol 1,4,5-trisphosphate; PtdIns-4,5-P_2: phosphatidylinositol 4,5-bisphosphate; DAG: diacylglycerol; EGTA: ethylene-bis(oxyethylenenitrilo)-tetraacetic acid. SAX:

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1,3,4,5-P_4 (15,16) raised the possibility that this conversion of the Ca^{2+}-mobilizing $InsP_3$ isomer to $InsP_4$ is relevant, in that it provides additional regulatory components of cellular Ca^{2+} homeostasis (17).

We have recently described a new metabolic pathway in adrenal glomerulosa cells by which Ins-1,3,4-P_3 is phosphorylated to a novel Ins-P_4 isomer (18). In the present study we provide additional data on the structural analysis of this new $InsP_4$ isomer, which indicates that it is the Ins-1,3,4,6-P_4 isomer of IP_4.

MATERIALS AND METHODS

Materials: Myo-[^{3}H]inositol, [^{3}H]inositol-1,4,5-P_3, [^{3}H]inositol-1,3,4,5-P_4 and [^{3}H]inositol-1,4-P_2 were obtained from New England Nuclear Corp.; collagenase, DNA-se (type I) were from Sigma Chemical Co.; and [Ile^5]-angiotensin II from Peninsula Labs. Amberlite MB 3A was from Aldrich Chemical Co. Culture media were prepared by the NIH Media Unit.

Preparation and incubation of cells: Bovine adrenal glomerulosa cells were prepared and cultured as described previously (18). Briefly, the outer 0.5 mm slices of bovine adrenal glands containing the glomerulosa tissue were minced and treated with collagenase followed by mechanical dispersion. Cells were plated in a density of 5 x 10^5/ml and kept in culture for 3 days. On the second day of the culture the medium was changed to one containing [^{3}H]inositol (20 µCi/ml) but no carrier inositol (see 18 for more details). After labelling for 24 hrs, cells were washed and preincubated at 37° C for 20 min before the addition of angiotensin II (50 nM). The reaction was terminated and inositol phosphates extracted as described previously (18). Samples were then applied to an HPLC column (Absorbosphere SAX, 5 µM, 250 mm, Alltech Applied Sci., Deerfield, IL) and eluted with a linear gradient of ammonium phosphate as detailed elsewhere (6).

Preparation of radiolabelled inositol phosphates:
During studies on the properties of an $InsP_3$-kinase preparation prepared from the adrenal cortex (19) by the method of Hansen (20), we observed that Ins-1,4,5-P_3 was rapidly converted to Ins-1,3,4,5-P_4, which was then dephosphorylated to Ins-1,3,4-P_3. The latter product was again phosphorylated to produce one of the new $InsP_4$ isomers that we observed in the stimulated glomerulosa cells. This reaction sequence produced almost exclusively the new $InsP_4$ isomer in prolonged incubations. Taking advantage of this property of our preparation, 2 µCi of [^{3}H] Ins-1,4,5-P_3 was incubated with 1.5 mg enzyme protein in 2.0 ml medium (50 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 5 mM Na-pyrophosphate, 5 mM ATP, 1 mM dithiothreitol, 10^{-5} M $CaCl_2$) which contained 0.2 µM Ins-1,4,5-P_3. Incubation was carried out for 4 hrs at 37 °C, and the reaction was stopped with 160 µl $HClO_4$ (60 %). After centrifugation, $HClO_4$ was extracted from the sample (21), which contained about 60% of its radioactivity as the new $InsP_4$. After purification on HPLC and desalting by sequential chromatography on Bio Rad AG 1X8 and Dowex W-50 (H^+), lyophylization gave 0.5-0.7 µCi of the new $InsP_4$ isomer, which was checked for purity by HPLC before periodate oxidation. Ins-1,3,4-P_3 was prepared from [^{3}H] Ins-1,3,4,5-P_4 (NEN, 1 Ci/mmol) by incubation with erythrocyte membrane 5-phosphatase (22).

Periodate oxidation: Periodate oxidation was performed according to (23) and (24) with slight modifications. [^{3}H] Ins-1,3,4-P_3 and [^{3}H] Ins-1,3,4,5-P_4, as well as the [^{3}H]labelled new $InsP_4$ isomer (0.1 µCi of each), were each separately incubated with 0.5 ml of 0.1 M $NaIO_4$ for 7-11 days in the dark at room temperature. Sodium-borohydrate (50 mg) was then added from an aqueous solution dropwise and the reaction was continued for 5-8 hours. The excess borohydrate was reacted with 1 N HCl until H_2 evolution ceased. Samples were then applied to 5 ml Sephadex G-15 columns (preswollen in 0.1 M phosphate buffer) and eluted with 0.1 M phosphate buffer. Fractions containing ^{3}H activity (2-4 ml) were pooled and diluted with
distilled water to 30 ml. $MgCl_2$ (1 mM final) and alkaline intestinal phosphatase (1000 IU, Sigma , Type VII-L) was added after the pH was adjusted to 10 with NaOH. The reaction was left to proceed for 6 hr at 37 °C. A mixture of unlabeled myo-inositol and altritol (prepared from altrose according to (23)) was added to each of the samples before lyophylization. The residue was distilled with 1% methanolic HCl and finally deionized with a mixed-bed ion-exchange resin (Amberlite, MB-3A, Aldrich Chemical Co., Milwaukee, Wis). The resulting polyols were separated by paper chromatography (23) as described by Irvine et al. (23). The strips were then bisected and one half was developed by the silver-nitrate method (25) with the modification of Frahn and Mills (26). The other half was cut into 1 cm pieces and analyzed for radioactivity in a β-spectrometer.

RESULTS AND DISCUSSION

Bovine adrenal glomerulosa cells prelabelled with [^{3}H]inositol for 24 hr contained mainly the two isomers of Ins-monophosphate, Ins-1-P and Ins-4-P, under basal conditions as shown on the HPLC elution profiles in Fig. 1. Basal levels of Ins-1,4,5-P_3 and Ins-1,4-P_2 were low, but the glomerulosa cells contained a larger peak which is assumed to be $InsP_5$ based upon its HPLC elution characteristics. Angiotensin II stimulated large increases in the levels of Ins-1,4,5-P_3 and Ins-1,3,4-P_3, together with even larger changes in Ins-1,4-P_2. The selective and early stimulation of Ins-4-P was followed by a delayed increase in the amount of Ins-1-P.

In addition to these well-characterized changes (18), three peaks were eluted in the $InsP_4$ region of extracts from AII-stimulated cells. The central peak increased rapidly upon stimulation with angiotensin II and was identified as the 1,3,4,5-tetrakisphosphate isomer described in other tissues (20,27-29). The other two peaks showed much slower responses and their levels increased continuously during AII stimulation. The first peak of this group was recently identified by us as a new $InsP_4$

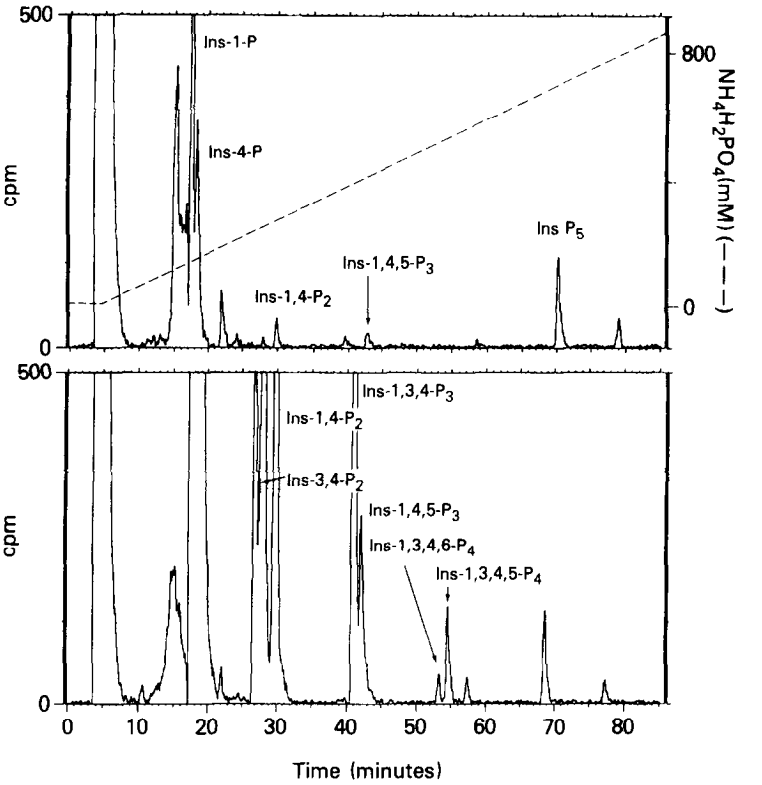

Fig. 1. HPLC elution profiles obtained from control (upper panel) or angiotensin II-stimulated (lower panel) glomerulosa cells. Cultured bovine adrenal glomerulosa cells were prelabelled with [^{3}H]inositol for 24 hrs, and after several washes they were stimulated with angiotensin II (50 nM) for 15 minutes. After extraction, inositol phosphates were resolved on a SAX HPLC column eluted with a linear gradient of ammonium phosphate (---). The results shown are representative of at least 15 similar observations.

isomer produced from Ins-1,3,4-P_3 by a kinase distinct from the 3-kinase which phosphorylates Ins-1,4,5-P_3 (18). Double label studies indicated that this isomer was neither a cyclic form nor a product of simple phosphate migration (18). Since it was produced from Ins-1,3,4-P_3, only two possibility remained for its structure: Ins-1,2,3,4-P_4 or Ins-1,3,4,6-P_4.

To distinguish between these two possibilities, we used the periodate oxidation method of Grado and Ballou (23). If the structure is Ins-1,2,3,4-P_4 there are two vicinal -OH groups (on positions 5 and 6); although these are in the trans position, periodate is able to open the inositol ring between them during long exposure as showed by Irvine et al. (24) in the case of Ins-1,3,4-P_3. On the other hand, Ins-1,3,4,6-P_4 is not susceptible to periodate oxidation because of the lack of vicinal

-OH groups (similar to Ins-1,3,4,5-P_4). After periodate oxidation and reduction of the aldehyde groups by Na-borohydrate, the phosphate groups were hydrolyzed and the resulting polyols were analyzed by paper chromatography (24). These experiments revealed that under conditions where Ins-1,3,4-P_3 was converted to altritol, both Ins-1,3,4,5-P_4 and the new $InsP_4$ isomer gave inositol as the final product. The resistance of this $InsP_4$ isomer to periodate oxidation indicates that its structure is Ins-1,3,4,6-P_4. These results are consistent with the recent finding of Shears et al. (30) that Ins-1,3,4-P_3 is phosphorylated to a new $InsP_4$ isomer in liver homogenate. They found this isomer to be resistant to periodate oxidation, suggesting that its structure is Ins-1,3,4,6-P_4. Our results did not reveal the structure of the third peak eluting immediately behind Ins-1,3,4,5-P_4, which is very likely to be another $InsP_4$ isomer.

The physiological importance of the complex series of reactions by which Ins-1,4,5-P_3 is transformed to other products through phosphorylations-dephosphorylations is not yet clear. The discovery of the inositol-tris-tetrakisphosphate pathway provided an alternative mechanism of Ins-P_3 elimination, but also raised the possibility that this metabolic route produces additional molecules with potential messenger functions (17). Our finding that Ins-1,3,4-P_3 is also phosphorylated to another Ins-P_4 isomer, now identified as Ins-1,3,4,6-P_4 in angiotensin-stimulated cells, indicates that there is a more complex metabolic network for the disposition of inositol polyphosphates. We have also observed that Ins-1,3,4,6-P_4 can be converted to $InsP_5$ by adrenocortical cytosol (Guillemette et al. submitted for publication). In this way, Ins-1,3,4,6-P_4 might be the link between $InsP_3$-s and $InsP_5$, a compound already shown to be present in avian erytrocytes (31) and mammalian tissues (29,32). However, the reason why cells expend considerable energy to modify the structure of the inositol-polyphosphates produced during hormonal activation by agonist ligands remains to be determined.

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