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ANGIOTENSIN-INDUCED FORMATION AND METABOLISM OF INOSITOL POLYPHOSPHATES IN BOVINE ADRENAL GLOMERULOSA CELLS

Gaetan Guillemette, Albert J. Baukal, Tamas Balla, and Kevin J. Catt

Endocrinology and Reproduction Research Branch,
National Institute of Child Health and Human Development,
National Institutes of Health, Bethesda, MD 20892

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Summary: The actions of angiotensin II (AII) on inositol polyphosphate production and metabolism were analyzed in cultured bovine adrenal glomerulosa cells. In cells labeled for 24 hr with [^{3}H]inositol, AII caused a rapid and prominent rise in formation of Ins-P_3 (mainly the Ins-1,3,4,-P_3 isomer) and of Ins-P_4, with marked increases in two isomers of Ins-P_2 and Ins-P. These findings are consistent with rapid formation and turnover of Ins-1,4,5-P_3, partly via conversion to Ins-1,3,4,5-P_4 with subsequent metabolism to Ins-1,3,4-P_3 and lower inositol phosphates. The demonstration of a cytosolic Ins-P_3-kinase gave further evidence for the presence of the tris/tetrakisphosphate pathway and Ins-P_4 synthesis during AII action in the bovine adrenal cortex.

Increased turnover of polyphosphoinositides is generally regarded as the primary mechanism by which many hormones elicit calcium-mediated metabolic and secretory responses in their target tissues (1-3). The major link between inositol phospholipid turnover and intracellular Ca^{2+} mobilization appears to be Ins-1,4,5-P_3, derived from cleavage of the plasma membrane lipid precursor, PtdIns-4,5-P_2, by phospholipase C. Following the recognition of Ins-1,4,5-P_3 as a major mediator of calcium mobilization, another Ins-P_3 isomer identified as Ins-1,3,4-P_3 was found in parotid gland (4) and other hormone-stimulated tissues (5-8). The origin and significance of the latter compound were clarified by recent discovery of a new metabolic pathway (9-11) through which Ins-1,4,5-P_3 is converted to Ins-1,3,4,5-P_4, a compound originally found in brain

The abbreviations used are: Ins, inositol; Ins-1,4,5-P_3, inositol-1,4,5-trisphosphate; Ins-P_4, inositol tetrakisphosphate; AII, angiotensin II; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; TCA, trichloroacetic acid.

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cortical slices (12). The subsequent dephosphorylation of Ins-1,3,4,5-P_4 can then produce the inactive metabolite, Ins-1,3,4-P_3, for which no biological role has been established. In the adrenal cortex, angiotensin II stimulates aldosterone secretion by a calcium-dependent mechanism (13) that involves the formation of Ins-P_3 (14,15). We have recently described an intracellular receptor for Ins-1,4,5-P_3 in the bovine adrenal cortex (16) with a potential role in Ca^{2+} mobilization during AII action (17). In the present study, we show that AII increases the formation of both Ins-P_3 isomers and Ins-P_4 in cultured bovine adrenal cells, and that Ins-1,4,5-P_3 is converted to Ins-P_4 by a partially purified cytosolic kinase from the bovine adrenal cortex.

Material and Methods

Materials: myo-[^{3}H]inositol, [^{3}H]Ins-1,4-P_2, [^{3}H]Ins-1,4,5-P_3 were obtained from New England Nuclear Corp., [^{14}C]Ins-1-P from Amersham International; collagenase and DNase-I from Sigma Chemical Co.; and [Ile^5]angiotensin II from Peninsula Labs. Culture media were prepared by the NIH Media Unit.

Cells preparation: Bovine adrenal glomerulosa cells were prepared by collagenase digestion of the outer 0.5 mm layers (containing the zona glomerulosa) of bovine adrenal glands (18). The tissue was minced into $\sim$1 mm^2 fragments and incubated at 37°C for 20 min in medium 199 containing: collagenase 2 mg/ml, DNase-I 0.2 mg/ml and a mixture of antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). Enzymatic digestion of 3-4 g of tissue/250 ml medium was repeated 5 times, and at the end of each digestion the tissue was further disrupted by aspiration through a wide-mouthed pipet. The cells from digestions 2-5 were combined and plated at a density of 500,000 cells/ml in DMEM culture medium supplemented with horse serum (10%), fetal bovine serum (2%), and antibiotics. The cells were cultured for 4 days at 37°C and the culture medium was changed daily.

Cell labeling and stimulation: On the third day of culture, the cells were incubated in a medium containing myo-[^{3}H]inositol (20 µCi/ml). After 24 hours, cells were washed free of [^{3}H]inositol and incubated at 37°C in the presence or absence of AII (10 nM). Incubations were terminated with 200 µl of ice-cold 10% $HClO_4$ and the cells were scraped free and transferred to plastic tubes (Eppendorf) containing 100 µl of 10 mM EDTA. Samples were neutralized with 300 µl of a Freon: tri-n-octylamine (1:1) mixture, with vigorous mixing followed by centrifugation. The upper phase (containing the water-soluble products) was analyzed by strong anion exchange (SAX) chromatography on a 4.6 x 250 mm Adsorbosphere (5 µ) column, with elution by a linear gradient of 0-0.7 M ammonium phosphate (pH 3.35) from 5 to 75 minutes, and inositol phosphates were identified by coelution with labeled standards. SAX columns rapidly lose resolution capacity when eluted with high ionic strength solutions; this does not affect the elution sequence of the individual inositol phosphates but shortens their elution times, as can be seen from the different elution times of Ins-P_4 in Fig. 1 and Fig. 2. It also impairs the separation of closely eluting compounds (as it can

be seen by the different elution profiles for Ins-P_3 peaks in Fig. 1 and the inset of Fig. 1).

Inositol trisphosphate kinase preparation: Ins-P_3-kinase was partially purified from bovine adrenal cortex by the procedures of Hansen et al (19). The tissue was homogenized (1:10, w/v) in 0.32 M sucrose, 10 mM Hepes/KOH, pH 7.3, 1 mM EGTA, 2 mM $MgCl_2$ and 2 mM dithiothreitol in a Dounce homogenizer (10 strokes loose pestle). After a centrifugation at 25,000 for 10 min, the supernatant was recentrifuged at 100,000 g for 90 min. The last supernatant was fractionated with ammonium sulfate and a 23-40% ammonium sulfate fraction was dialyzed overnight against 10 mM Tris/HCl pH 7.0, 2 mM $MgCl_2$ and 2 mM dithiothreitol at 4°C.

Inositol trisphosphate kinase assay: Ins-P_3-kinase activity was measured in a buffer containing 50 mM Tris/HCl, pH 8.0, 5 mM ATP, 5 mM $MgCl_2$, 5 mM sodium pyrophosphate and 1 mM dithiothrietol. The partially purified enzyme (0.2 - 2 mg of protein) was incubated at 37°C for different periods of time in the presence of [^{3}H]Ins-P_3 (0.1 - 10 µM). The incubation was stopped in ice-cold 10% TCA and after 5 washes with diethylether the inositol phosphates were analyzed by HPLC.

Results

Incubation of cultured bovine adrenal glomerulosa cells with 10 nM AII stimulated the production of several inositol phosphates, as shown by the HPLC elution profiles in Fig. 1. The most prominent effect of AII is the substantial increase in two isomers of $InsP_2$, one corresponding to Ins-1,4-P_2 and another that probably represents Ins-1,3-P_2 or Ins-3,4-P_2. As shown in Table 1, these compounds are increased from undetectable levels to 7000 cpm. The production of two isomers of Ins-P (Ins-1-P and Ins-4-P) is also substantially increased, especially that of Ins-4-P, from <1000 cpm to 17,000 cpm. The production of Ins-P_3 was increased by 4-fold after 5 minutes of stimulation. A detailed analysis of the Ins-P_3 peak shown in Figure 1 (inset) revealed that it consisted of two isomers, the amount of Ins-1,3,4-P_3 far exceeding that of Ins-1,4,5-P_3. A finding of major interest was the consistent production of a metabolite with the elution properties of inositol tetrakisphosphate. This compound increased in a time-dependent fashion upon AII stimulation, rising from undetectable levels to 433 cpm, in proportion with the increase in Ins-P_3. In contrast, a more highly acidic compound with elution properties corresponding to Ins-P_5 did not show any significant change during hormonal stimulation.

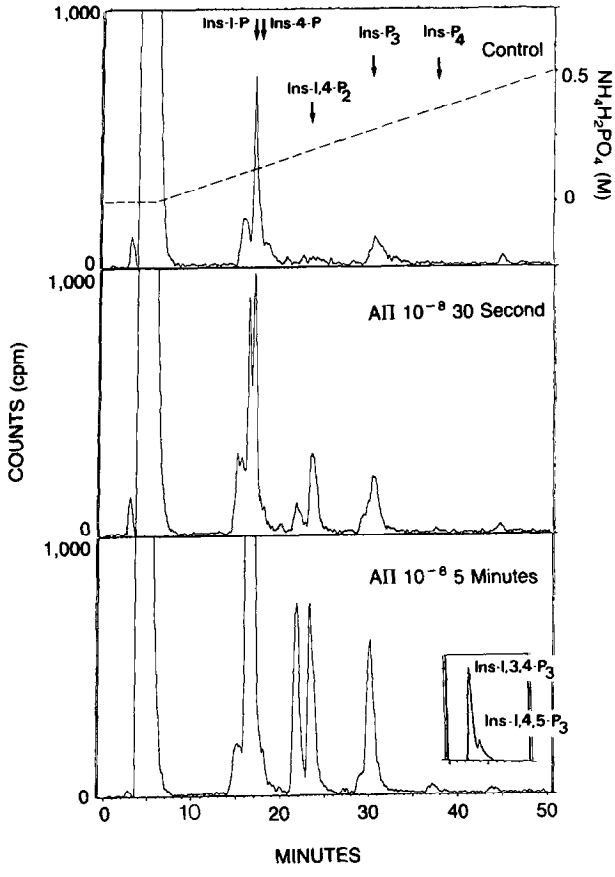

Figure 1. High performance liquid chromatography analysis of inositol phosphates derived from bovine adrenal glomerulosa cells. After a pre-labeling period of 24 hours with [^{3}H]inositol, the cells were stimulated with 10 nM AII for 30 sec (middle panel) and 5 min (lower panel). In the upper panel (control without AII) the arrows indicate the elution times of radioactive standards. These profiles are representative of two identical experiments performed in duplicate.

In order to ascertain the identity of Ins-P_4 and to verify the presence of the enzyme responsible for its production in the bovine adrenal cortex, we assayed adrenal cytosol for Ins-P_3-kinase activity as described by Hansen et al (19). As shown in Figure 2, the partially purified enzyme preparation actively converted [^{3}H]Ins-P_3 to [^{3}H]Ins-P_4. Furthermore, the accumulation of Ins-P_4 with only a very small increase in Ins-P_2 indicates that the cytosolic preparation is essentially devoid

TABLE 1. Effects of AII on formation of inositol phosphates in bovine adrenal glomerulosa cells

Compound	Control	30 sec	5 min
Ins-1-P	5455[1]	4192	8988
Ins-4-P		6127	16910
Ins-3,4-P_2 (?)	ND	1181	5947
Ins-1,4-P_2	ND	3120	6770
$InsP_3$	1568	3560	7269
$InsP_4$	ND	275	444
$InsP_5$	301	320	392

Data are the amounts (cpm) of [^{3}H]inositol phosphates formed during stimulation of prelabeled glomerulosa cells with 10 nM AII. Each value is the mean of closely agreeing duplicates (±10%). ND: not dectectable This result is representative of at least 3 similar experiments.

[1]The amount of Ins-4-P in non-stimulated samples is very low and was not clearly resolved from the Ins-1-P peak which represents most of the radioactivity under control conditions. Therefore, this value is predominantly due to Ins-1-P, with a small amount (<20%) of Ins-4-P (seen as the minor shoulder in Fig. 1, upper panel).

of Ins-P_3-phosphatase, consistent our previous observation (17) that the Ins-P_3-phosphatase of bovine adrenal cortex is a particulate enzyme. The Ins-P_4 produced by phosphorylation of tritiated standard Ins-1,4,5-P_3 in the Ins-P_3-kinase assay coeluted during HPLC with the Ins-P_4 endogenously produced by AII-stimulated glomerulosa cells, confirming the identity of the latter metabolite.

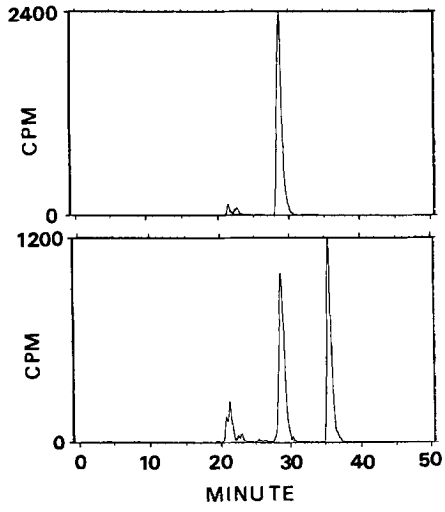

Figure 2. High performance liquid chromatography analysis of the products derived from the incubation of [^{3}H]Ins-1,4,5-P_3 (10 μM) with a cytosolic extract of the bovine adrenal cortex (0.3 mg protein). Samples were analyzed at zero time (upper panel) and after 5 min (lower panel). These profiles are representative of 5 similar experiments with 3 different adrenal cytosol preparations.

Discussion

The multiplicity of inositol polyphosphates and isomers detected by anion exchange HPLC during AII stimulation indicates the complexity of the metabolism of Ins-1,4,5-P_3 following its hormone-activated cleavage from phosphatidylinositol bisphosphate in the plasma membrane. The current finding of two isomers of Ins-P, Ins-P_2, and Ins-P_3, as well as the presence of Ins-P_4 and Ins-P_5, extend our recent observations on inositol phosphate production by rat glomerulosa cells (6). In the latter report, rapid formation of Ins-1,4,5-P_3 and Ins-1,3,4-P_3 were demonstrable, as well as selective metabolism to Ins-4-P rather than Ins-1-P during the onset of stimulation of AII. The preferential formation of Ins-4-P indicates that stimulated polyphosphoinositide catabolism proceeds mainly via the 4-monophosphate metabolite, and also that direct hydrolysis of phosphatidylinositol is not an early response to hormone stimulation.

In rat glomerulosa cells, which were labeled for 3 hrs with [^{3}H]inositol, angiotensin II-stimulated increases in the levels of both isomers of Ins-P_3 were relatively small and were not associated with detectable production of Ins-P_4 (6). However, during the present study on cultured bovine glomerulosa cells labeled with [^{3}H]inositol for 24 hrs, AII-induced formation of Ins-P_3 was much more prominent and was associated with the appearance of a compound that co-eluted with our Ins-P_4 standard. This difference may be related to the lengths of the labeling periods or to a species difference in the rates of production and/or degradation of Ins-P_3 and Ins-P_4. In bovine adrenal cells, the presence of a cytosolic kinase able to phosphorylate Ins-1,4,5-P_3, together with the AII-stimulated formation of Ins-1,4,5-P_3 and its higher phosphorylated derivative (Ins-1,3,4,5-P_4) and the formation of a second Ins-P_3 isomer (Ins-1,3,4-P_3) presumably derived from 5'-dephosphorylation of InsP_4, provides compelling evidence for the existence of the inositol tris/tetrakisphosphate pathway. The presence of such a pathway was also suggested by the recent observation of an Ins-P_3 kinase activity in permeabilized adrenal cells (20).

The functional signficance of this pathway of Ins-P_3 metabolism is not yet clear: it might represent a second route (together with the 5'-phosphatase) for inactivation of Ins-1,4,5-P_3, or it may generate a second messenger that is responsible for one of the steps involved in stimulation of steroidogenesis. The presence of a new isomer of Ins-P_2, which may be either Ins-1,3-P_2 or Ins-3,4-P_2, was an additional feature of interest with present study, since it may represent a specific metabolite of Ins-1,3,4-P_3. The detailed analysis of the rates of appearance and disappearance of the multiple inositol phosphates and isomers generated during AII stimulation should provide a better understanding on the mechanisms through which AII increases steroid production in the adrenal cortex.

Acknowledgements

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