H.p.l.c. analysis of inositol monophosphate isomers formed on angiotensin II stimulation of rat adrenal glomerulosa cells

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[³H]Inositol-prelabelled isolated rat adrenal glomerulosa cells were stimulated with 25 nM-AII ([Asp¹, Ile⁵]-angiotensin II) in the presence of 10 mM-Li⁺, and the resulting inositol monophosphate isomers were separated successfully by using a recently developed h.p.l.c. methodology. Two major peaks of radioactivity were detected which showed the same retention characteristics on h.p.l.c. as inositol 4-phosphate and inositol 1-phosphate and which increased 5-fold and 8-fold respectively on stimulation with AII. In addition, a relatively small peak with the retention characteristics of inositol 1:2-cyclic phosphate was seen to undergo a 1.5-fold increase on stimulation. This was not considered sufficient to suggest that cyclic phosphoinositols were a major product of AII-stimulated phosphoinositide turnover. No peaks of radioactive material were detected in the regions expected for inositol 2-phosphate (an acid hydrolysis product of inositol 1:2-cyclic phosphate) or inositol 5-phosphate. These results establish the identity of the major inositol phosphate products in AII-stimulated glomerulosa cells and confirm and extend the previous observations of Balla, Baukal, Guillemette, Morgan & Catt [(1986) *Proc. Natl. Acad. Sci.* 83, 9323–9327].

INTRODUCTION

The increased turnover of the polyphosphoinositides through the activation of hormone-sensitive phospholipase C in response to stimulation by a Ca²⁺-mobilizing hormone is a widely observed phenomenon [for review see Berridge & Irvine (1984)]. In rat adrenal glomerulosa cells, a number of observations (Farese *et al.*, 1984; Enyedi *et al.*, 1985; Balla *et al.*, 1986) have been made suggesting that such a mechanism is activated in response to the Ca²⁺-mobilizing hormone, [Asp¹, Ile⁵]-angiotensin II (AII). These include the demonstration of the rapid breakdown of PtdIns4P and PtdIns(4,5)P₂ with a correspondingly rapid rise in InsP₃ and InsP₂ (Enyedi *et al.*, 1985) that occurs in the absence of stimulation of cyclic AMP formation (Bell *et al.*, 1981). Further work by Balla *et al.* (1986) has confirmed the results of Enyedi *et al.* (1985) and extended them by demonstrating that the InsP₃ isomer accumulating in the first few seconds of stimulation is Ins(1,4,5)P₃, which is known to possess Ca²⁺-mobilizing ability [for review, see Berridge & Irvine (1984)], whereas that isomer which shows a sustained accumulation in the presence of lithium is Ins(1, 3, 4)P₃. Those authors present further evidence that two isomers of InsP are formed on stimulation, and the identities of these isomers are suggested to be Ins1P and Ins4P. Although such a suggested identity is reasonable in view of the identification of these two isomers in liver (Storey *et al.*, 1985) and in vasopressin-stimulated platelets (Siess, 1985), it is by no means certain that this is also the case in the glomerulosa cell. Firstly the separation achieved for the two isomers using the h.p.l.c. system reported by Balla

et al. (1986) is poor, so that identification, especially of Ins4P, is uncertain, and it is not clear whether these are the only products. Moreover, although those authors used acidic and neutral extraction techniques to show that Ins2P was not a major product, it was not possible to show whether it might have been present as a minor product. Secondly, a recent report has demonstrated the formation of Ins5P in brain (Ackermann *et al.*, 1987). In view of the importance of a knowledge of the identity of these isomers to our understanding of phosphoinositide and phosphoinositol metabolism in the glomerulosa cell (Balla *et al.*, 1986), we have attempted a more rigorous identification of these products using a specially developed h.p.l.c. system utilizing a shallow salt gradient. By using this approach we were able to investigate the possible formation of Ins[1,2(cyc)]P as well as the inositol 1-, 2-, 4- and 5-monophosphate isomers and so clarify the results described by Balla *et al.* (1986).

MATERIALS AND METHODS

Materials

Female Sprague–Dawley rats were obtained from Olac. Collagenase (from *Clostridium histolyticum*) and DNAase (deoxyribonuclease 1) were obtained from Boehringer Mannheim. AII was the W.H.O. standard (A, 70/302). Dowex resin (Cl[−] form, 1X10, 200–400 mesh) was supplied by Dow Chemicals, Toronto, Canada. Partisil SAX-10 packing material and all h.p.l.c. column fittings were supplied by HPLC Technology Ltd. (Macclesfield, Cheshire SK11 6PJ, U.K.). Ins2P (dicyclohexylamine salt) was obtained from Sigma, whereas

Abbreviations used: DG, diacylglycerol; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; Ins, inositol; GroPIns, glycerophosphoinositol; Ins1P, inositol 1-phosphate; Ins2P, inositol 2-phosphate; Ins4P, inositol 4-phosphate; Ins5P, inositol 5-phosphate; Ins[1:2(cyc)]P, inositol 1:2-cyclic phosphate; Ins(1,4)P₂, inositol 1,4-bisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4)P₃, inositol 1,3,4-trisphosphate; AII, [Asp¹, Ile⁵]angiotensin II.

myo-[^3H]inositol, *myo*-[^3H]inositol 1,4-bisphosphate, *myo*-[^3H]inositol 1,4,5-trisphosphate and *myo*-[^{14}C]inositol 1-phosphate were supplied by Amersham International. [^{32}P]inositol 1,4-bisphosphate (prepared from erythrocyte ghosts) was kindly donated by M. Salmon (St. Georges Hospital Medical School, London SW17 0RE, U.K.). All liquid-scintillation counting was carried out on either a Beckman LS7500, or an LKB 1217 'Rackbeta', liquid-scintillation counter, Optiphase X being used as a scintillant.

Cell preparation and incubation

Adrenal glands were excised from 25 female Sprague-Dawley rats (180–220 g), and adrenal glomerulosa cells were isolated by the method of Hanning *et al.* (1970), with the following modifications. The capsules were defatted and incubated in Krebs–Ringer bicarbonate buffer (pH 7.4)/11 mM-glucose/0.5% (w/v) human serum albumin (KRBGA) containing 2 mg of collagenase/ml. After 60 min incubation, the capsules were physically disrupted, in the presence of 4 μg of DNAase/ml, by repeated aspiration using a 5 ml Gilson Repetman pipette. Isolated cells were washed three times and resuspended in 1.5 ml of KRBGA with the addition of 50 μCi of [^3H]inositol/ml. They were then incubated for a further 3 h in order to allow incorporation of the ^3H label into inositol-containing phospholipids. The labelled cells were washed and resuspended in fresh KRBGA containing 10 mM-LiCl as a replacement for NaCl. The cell preparation was more than 95% pure as judged by optical-microscopic cell count and was contaminated with less than 5% adrenal fasciculata-reticularis cells. Cell viability was more than 98% (by Trypan Blue exclusion). Cell yield was, on average, 375 000 per adrenal gland. After incubation (0.45 ml portion) in silane-treated glass tubes for 15 min, AII was added (50 μl) to a final concentration of 25 nM; 50 μl of KRBGA was added to control incubations. Stimulation was terminated, after 30 min, by extraction with 1.8 ml of chloroform/methanol/X (100:200:1, by vol), where X is conc.HCl or water for acid or neutral extraction respectively. Phase separation was achieved by addition of 0.625 ml of chloroform and a further 0.625 ml of 0.1 M-HCl (acid extraction) or water (neutral extraction). After brief centrifugation to complete phase separation, 1.8 ml of the aqueous phase was removed (and neutralized with NaOH in the case of acid extraction) and stored at -18°C . Each experiment was performed in triplicate. All incubations were carried out at 37°C under an O_2/CO_2 (19:1) atmosphere.

Dowex-column chromatography before h.p.l.c. analysis

The aqueous phase from the solvent extractions was made 1 mM with respect to EDTA, and applied to a 1 ml (Cl^- form) column. The column was then washed with 2.5 ml of water, and a 20 ml linear gradient of 0–400 mM-LiCl was applied; fractions (1.3 ml) were collected throughout the procedure. Peaks were located by determining the radioactivity in a 100 μl sample from each fraction by liquid-scintillation counting. This method allows rapid separation of the phosphoinositols into inositol mono-, bis- and tris-phosphates. Fractions were pooled from the beginning of the gradient (fraction 3) to the end of the peak that was eluted in the same region as [^{14}C]Ins1P standard (fraction 8), as indicated in Fig. 1 (below). Approximate elution concentrations

determined by using [^3H]GroPIns, [^{14}C]Ins1P, [^{32}P]Ins-(1,4) P_2 and [^3H]Ins(1,4,5) P_3 as calibration standards were 50, 100, 230 and 300 mM-LiCl respectively (results not shown). Each sample was processed in this way in approx. 35 min. Columns were used up to six times, being regenerated with 1 M-HCl and washed to neutrality with water between samples. Each column was calibrated with [^{14}C]Ins1P before use.

H.p.l.c. separation of inositol monophosphate isomers

In order to prepare samples for h.p.l.c. analysis, pooled fractions eluted from the Dowex columns were freeze-dried in the presence of 0.5 mg of Ins2P and washed twice with 15 ml of 100% ethanol. The ethanol-insoluble material was dried under a slow stream of N_2 and redissolved in 1 mM-EDTA (1 ml). Aliquots (50 μl) were removed for recovery determination (> 80%). All h.p.l.c. separations were carried out on a 15 cm $\times$ 0.5 cm Partisil SAX 10 column preceded by a 5 cm $\times$ 0.5 cm guard column of the same material. Solvent delivery was by an Altex high-pressure mixing gradient system. Flow rate was maintained at 1.3 ml/min throughout the entire procedure. After injection of the sample, the column was eluted for 5 min with water. A linear gradient from 0 to 300 mM-ammonium formate (buffered to pH 5.0 with H_3PO_4) was then applied over a 24 min period. Elution with 300 mM-ammonium formate, pH 5.0, was maintained for a further 6 min before returning to elution with water only over a 2 min period. The column was then washed with water for 12 min before injection of the next sample. Samples were applied in 2 ml total volume of 1 mM-EDTA containing cyclic AMP and AMP, and sample binding and column performance were monitored at 254 nm throughout the procedure. Fractions were collected at 2 min intervals for the first 12 min, and then at 0.25 min intervals for the duration of the gradient. Radioactivity in each sample was determined by liquid-scintillation counting.

In order to obtain maximum reproducibility from this method, the column was pre-run once with a blank sample of cyclic AMP and AMP in 1 mM-EDTA before injection of tissue extracts or standards. After eight such injections, the column was cleaned by running the same gradient profile using 1 M-ammonium formate, pH 3.7, instead of 300 mM-ammonium formate, pH 5.0.

Preparation of radioactive standards for h.p.l.c.

A ^{14}C -labelled mixture of Ins1P, Ins2P and Ins4P was prepared by the method of Tomlinson & Ballou (1961). Briefly, 1 mg of Ins2P and 0.5 μCi of [^{14}C]Ins1P were heated for 30 min at 100°C in 1 M-HCl. The time course of this reaction was analysed by h.p.l.c. and, by comparison with the results obtained by Tomlinson & Ballou (1961), who used paper chromatography, it was possible to identify the retention times of the different monophosphate isomers (for further details, see the Results section).

^3H -labelled mixtures of Ins1P and Ins4P, or Ins1P, Ins4P and Ins5P, were prepared by alkaline hydrolysis (38 h, 110°C) of [^3H]Ins(1,4) P_2 or [^3H]Ins(1,4,5) P_3 respectively by the method of Tomlinson & Ballou (1961). The inositol monophosphate products were recovered by using the Dowex chromatography procedure described above, and LiCl was removed by an ethanol wash before separation by h.p.l.c.

Fig. 1. Dowex chromatography of tissue extracts

Adrenal glomerulosa cells, prelabelled with [^3H]inositol, were incubated with (●) or without (○) AII (25 nM, 30 min) and extracted using acidic (a) or neutral (b) extraction procedures as described in the text. The aqueous phases were transferred to Dowex (Cl^- form) columns, which were subsequently eluted with a linear gradient of 0–400 mM-LiCl (—). Fractions (1.3 ml) were collected and radioactivity determined in a 100 μl aliquot from each. The elution positions of [^{14}C]Ins1P and [^{32}P]Ins(1,4) P_2 radioactive (*) standards are indicated (▼)

[^3H]GroPIns and [^{14}C]Ins[1:2(cyc)]P were prepared as described previously (Carter *et al.*, 1986).

RESULTS

Separation of phosphoinositols on Dowex

Elution of the aqueous tissue extracts from Dowex resin with LiCl gave rise to two major peaks, both of which were enhanced by AII stimulation, as shown in Fig. 1, which shows the profiles from acidic (Fig. 1a) and neutral (Fig. 1b) extractions. The material eluted in the region of 50–100 mM-LiCl (peak 1) co-migrated with the [^{14}C]Ins1P standard. This peak was pooled, as indicated in Fig. 1, for further analysis by h.p.l.c.. Additional material (peak 2) was seen to be eluted in the same region as the [^{32}P]Ins(1,4) P_2 standard (250 mM-LiCl) and is suggested to be Ins P_2 of unknown isomerism. This is in agreement with the observation of Enyedi *et al.* (1985), who demonstrated accumulation of inositol mono-, bis- and tris-phosphate-like material in adrenal glomerulosa cells stimulated with AII in the presence of 10 mM-LiCl.

Acidic extraction (Fig. 1a) recovered at least twice the radioactivity relative to the neutral extraction (Fig. 1b) for both stimulated and unstimulated samples.

Fig. 2. H.p.l.c. separation of radiolabelled inositol monophosphate standards

Mixtures of radiolabelled inositol monophosphate isomers prepared by treatment of [^{14}C]Ins1P with 1 M-HCl (100 $^{\circ}\text{C}$) for 10 min (○) and 30 min (●), were separated by h.p.l.c. as described in the Materials and methods section. The identification of peaks I, II, and III as [^{14}C]Ins1P, [^{14}C]Ins2P, and [^{14}C]Ins4P is described in the Results section. The separation of a mixture of ^3H -labelled mixture of Ins1P, Ins4P and Ins5P (■), prepared by alkaline hydrolysis of [^3H]Ins(1,4,5) P_3 (see the Materials and methods section) is also shown. By comparison with peaks I, II, and III, peak IV was identified as [^3H]Ins5P (see the Results section).

Determination of retention times of radioactive standards on h.p.l.c.

H.p.l.c. analysis (see Fig. 2) of the isomerization of [^{14}C]Ins1P in hot acid [as described by Tomlinson & Ballou (1961)], showed within 10 min a substantial conversion of the starting material (peak I) into a new material with a slightly longer retention time (peak II) (see Fig. 2). Prolonged heating (greater than 20 min, 100 $^{\circ}\text{C}$) of the reaction mixture gave rise to a further minor product (peak III) of even greater retention time (see Fig. 2).

By comparison with the results of Tomlinson & Ballou (1961), which were obtained by using descending paper chromatography, the rapidly formed product (peak II) was identified as Ins2P and the more slowly formed product (peak III) was identified as Ins4P. This difference in rate of formation is due to the greater relative ease of phosphate migration across vicinal *cis*-hydroxy groups (i.e. positions 1 and 2), than across vicinal *trans*-hydroxy groups (i.e. positions 1 and 4; see Tomlinson & Ballou, 1961). The identity of these peaks was confirmed, and the location of Ins5P achieved, by comparison with the inositol monophosphates produced by alkaline hydrolysis of [^3H]Ins(1,4) P_2 and [^3H]Ins(1,4,5) P_3 [giving mixtures of ^3H -labelled Ins1P and Ins4P, or Ins1P, Ins4P and Ins5P respectively (Tomlinson & Ballou, 1961)]. The comparative separation for the mixture of Ins1P, Ins4P and Ins5P (peaks I, II and IV respectively) is also shown in Fig. 2.

The separation of [^3H]inositol, [^3H]GroPIns, [^{14}C]Ins[1:2(cyc)]P and the mixture of ^{14}C -labelled Ins1P, Ins2P and Ins4P produced by prolonged (30 min) treatment of [^{14}C]Ins1P with hot acid is shown in Fig. 3.

Analysis of inositol monophosphates from tissue extracts

The h.p.l.c. separation of the inositol monophosphate isomers formed in the presence and absence of AII is

Fig. 3. H.p.l.c. separation of radiolabelled standards

Radiolabelled standards were separated on a Partisil SAX-10 column by using a linear gradient (—) of 0–300 mM-ammonium formate buffered to pH 5.0 with H_3PO_4 , and a flow rate of 1.3 ml/min. Fractions were collected throughout the gradient, and the radioactivity (○) in each fraction was determined by liquid-scintillation counting. The separation of [3H]GroPIns, [^{14}C]Ins[1:2(cyc)]P and [^{14}C]Ins1P/2P/4P (prepared as described in the text) by this method is as shown. ▼ Indicates the location of the radioactive (*) materials described. The elution positions of cyclic AMP and AMP, as detected at 254 nm, are also marked (▼). The retention time for these peaks was found to vary slightly between columns, but was reproducible for each column. This is illustrated by comparing the retention times for peaks I, II and III in Figs. 2 and 3, which were obtained using different columns.

Fig. 4. Effect of AII on Ins1P and Ins4P formation

[3H]Phosphoinositol products, recovered from AII-stimulated (●) and unstimulated (○) incubations using acid

shown in Figs. 4 and 5. By using this method of analysis it can be shown that the major inositol monophosphate products accumulating on AII stimulation in the presence of Li^+ are Ins1P and Ins4P (see Fig. 4). The mean increases in Ins1P and Ins4P were calculated to be $879 \pm 105\%$ and $514 \pm 63\%$ respectively of control mean for acidic extraction, and $737 \pm 48\%$ and $454 \pm 39\%$ respectively of control mean for neutral extraction. In all cases the increase was found to be highly significant, with $P < 0.0005$ by Student's t test (means and significance levels calculated from the results of two experiments, each performed in triplicate). In addition, AII stimulated the accumulation of Ins[1:2(cyc)]P ($148 \pm 6\%$ of control $P < 0.001$) as observed under neutral extraction conditions (see Fig. 5b). The identification of this material as Ins[1:2(cyc)]P is supported by its absence under acidic extraction conditions (see Fig. 5a) and by the absence of a detectable Ins2P peak in acid-extracted samples. To verify that this small quantity of Ins[1:2(cyc)]P recovered was not due to its degradation during the various chromatographic procedures, the [^{14}C]Ins[1:2(cyc)]P standard was subjected to elution from Dowex and subsequent removal of LiCl with ethanol, followed by analysis of the recovered material on h.p.l.c.. The material eluted as a single peak in the region of the untreated [^{14}C]Ins[1:2(cyc)]P standard (results not shown). This

(a), or neutral (b) extraction procedures, were prepared for h.p.l.c. as indicated in the Materials and methods section. Samples were then subjected to h.p.l.c. on Partisil SAX-10 as described in the text. The data are representative of those obtained from two separate experiments, each performed in triplicate, which gave similar results. The elution times of the ^{14}C -labelled radioactive (*) standards are indicated (▼).

Fig. 5. Effect of AII on Ins[1,2(cyc)]P formation

[^3H]Phosphoinositol products, recovered from AII-stimulated (●) and unstimulated (○) incubations using acid (a), or neutral (b) extraction procedures, were prepared for h.p.l.c. as described in the Materials and methods section. Samples were then subjected to h.p.l.c. on Partisil SAX-10 as described in the text. The data are representative of those obtained from two separate experiments, each performed in triplicate. The elution times of [^3H]GroPIns and [^{14}C]Ins[1,2(cyc)]P radioactive (*) standards are indicated (▼). Radioactivity in all fractions was determined from 5 min counts in a liquid-scintillation counter.

result is consistent with the absence of any detectable Ins2P peak under acidic extraction conditions (see Fig. 4a). No significant peak of material was detected in the region expected for Ins5P under acid or neutral extraction conditions, although the presence of this isomer cannot be ruled out by these results alone.

One surprising result was the poorer recovery of GroPIns, but greater recovery of InsP, by the acidic extraction compared with the neutral extraction (see Figs. 4 and 5). The phosphomonoester groups of the inositol monophosphates are known to be extremely resistant to acid hydrolysis (Pizer & Ballou, 1959). However, the phosphodiester group of GroPIns may not be so resistant to acid attack, so leading to partial degradation during extraction in acid. Alternatively, this peak may consist of more than one labelled compound, only one of which is acid-labile. However, this is unlikely, as the only known compound with similar retention time to GroPIns is Ins[1,2(cyc)]P, and this is clearly resolved from GroPIns by using this improved h.p.l.c. method (see Fig. 3).

DISCUSSION

These results demonstrate that there is substantial accumulation, in the presence of Li^+ , of Ins4P as well as Ins1P in the rat adrenal glomerulosa cell on stimulation with AII. This is consistent with the observations that Ins1P and Ins4P accumulate in platelets stimulated with vasopressin (Siess, 1985), and confirms the proposal of Balla *et al.* (1986) that Ins1P and Ins4P are the major inositol monophosphate products of AII stimulation. In addition, the observed increase in Ins4P to 100% of control values suggests firstly that Li^+ ions inhibit the breakdown of Ins4P as well as Ins1P in this tissue [as suggested by the observation of Balla *et al.* (1984) on Li^+ inhibition of ^{32}P or [^3H]arachidonic acid incorporation into PtdIns, and in agreement with the observations of Balla *et al.* (1986)]. This contrasts with the situation described by Storey *et al.* (1985) in liver, where only Ins1P dephosphorylation is sensitive to inhibition by Li^+ . Secondly, this large accumulation of Ins4P verifies that a substantial degradation of PtdIns4P and PtdIns(4,5) P_2 occurs on AII stimulation.

No measurable quantities of Ins2P or Ins5P are detected by acidic or neutral extraction, suggesting that Ins1P and Ins4P are the only major inositol monophosphate products. However, a small quantity of Ins[1,2(cyc)]P is detectable on neutral extraction and demonstrates an AII-stimulated accumulation. The presence of Ins[1,2(cyc)]P suggests the possible formation of cyclic derivatives of Ins(1,4,5) P_3 and Ins(1,4) P_2 by hormone-sensitive phospholipase C [as demonstrated *in vitro* with purified soluble phospholipase C (Wilson *et al.*, 1985)], which are then dephosphorylated to Ins[1,2(cyc)]P (Conolly *et al.*, 1986). Alternatively the direct breakdown of PtdIns in response to stimulation may have occurred as observed in caerulein-stimulated mouse pancreatic minilobules (Dixon & Hokin, 1985). It is not possible to determine which of these pathways is in operation on the basis of our present results alone. What is clear, however, is that the quantity of Ins[1,2(cyc)]P formed is extremely small, and so these pathways are probably not major alternative routes to phosphoinositol metabolism in the rat adrenal glomerulosa cell.

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