Inositol phosphate release and steroidogenesis in rat adrenal glomerulosa cells

Comparison of the effects of endothelin, angiotensin II and vasopressin

Elizabeth A. WOODCOCK,* Peter J. LITTLE and Jennifer K. TANNER

Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

Endothelin has steroidogenic activity in adrenal glomerulosa cells, as do two other vasoconstrictor peptides, angiotensin II and vasopressin. The steroidogenic activities of angiotensin II and vasopressin are probably mediated via the phosphatidylinositol-turnover pathway and associated changes in cytosolic Ca^{2+} concentration. Endothelin caused a steroidogenic response, which was small compared with that to angiotensin II and quantitatively similar to the vasopressin response. Cytosolic free Ca^{2+} responses were similarly higher to angiotensin II than to either of the other two peptides. However, total inositol phosphate responses to endothelin and angiotensin II were similar when these were measured over 20 min, and were quantitatively greater than the vasopressin response. A detailed study has been made of the phosphatidylinositol-turnover response to endothelin in comparison with responses to angiotensin II and vasopressin. Each of the three peptides produced a rapid and transient rise in $\text{Ins}(1,4,5)\text{P}_3$ (max. 5–15 s), followed by a slow sustained rise. $\text{Ins}(1,4,5)\text{P}_3$ was metabolized by both dephosphorylation and phosphorylation pathways, but the relative importance of the two metabolic pathways was different under stimulation by each of the three peptides. These findings show that adrenal glomerulosa cells can distinguish between the stimulation of phosphatidylinositol turnover by three different effectors. These differences in the pathway may be associated with the observed different steroidogenic and Ca^{2+} responses to the three peptides.

INTRODUCTION

Responses to many different hormones and neurotransmitters are initiated by activation of the phosphatidylinositol (PtdIns) turnover pathway. This pathway involves the receptor-activated hydrolysis of a plasma-membrane lipid, phosphatidylinositol 4,5-bisphosphate. This yields $\text{Ins}(1,4,5)\text{P}_3$, which releases intracellular Ca^{2+} [1,2], and *sn*-1,2-diacylglycerol (DAG), which activates protein kinase C [3]. In most cells $\text{Ins}(1,4,5)\text{P}_3$ is subject to metabolism via dephosphorylation to $\text{Ins}4\text{P}$ as well as via phosphorylation to $\text{Ins}(1,3,4,5)\text{P}_4$. $\text{Ins}(1,3,4,5)\text{P}_4$ by further phosphorylation and dephosphorylation reactions generates a considerable number of inositol phosphate products [4]. The significance of this complex metabolism is not yet fully understood, but $\text{Ins}(1,3,4,5)\text{P}_4$ has been implicated in the control of the entry of extracellular Ca^{2+} [5], the redistribution of intracellular Ca^{2+} stores [6,7] and $\text{Ins}(1,4,5)\text{P}_3$ metabolism [8] and thus might be expected to influence cellular responses. The enzymes that metabolize $\text{Ins}(1,4,5)\text{P}_3$, i.e. $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase and $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase, are subject to regulation [9–12], and thus presumably the metabolic fate of $\text{Ins}(1,4,5)\text{P}_3$ can be controlled. The relative importance of the two pathways of metabolism of $\text{Ins}(1,4,5)\text{P}_3$ appear to be different in different cell types [13,14], but whether such differences in inositol phosphate metabolism can occur within the one cell type under different stimulatory conditions has not been addressed. Differences in DAG profiles, however, have been reported in the one cell type under stimulation by different effectors [15,17].

Endothelin (ET) is a newly described peptide released from the vascular endothelium [18]. It was identified initially as a potent vasoconstrictor, but has since been shown to stimulate a number of other responses, including inotropic responses in atria [19,20], release of atrial natriuretic peptide [21], mitogenesis in a number of different cell types [17,22] and steroidogenesis in adrenal

glomerulosa cells [23,24]. The vasoconstrictor effects of ET were initially ascribed as being due to a direct opening of voltage-dependent Ca^{2+} channels [25]. However, subsequent data showed that ET stimulated release of $\text{Ins}(1,4,5)\text{P}_3$ and release of intracellular Ca^{2+} in vascular smooth-muscle cells [26], mechanisms which are similar to those described for many other vasoconstrictors, including angiotensin II (AII), vasopressin (VP) and α -adrenergic agonists [27–29]. However, detailed analysis of the responses to ET and other vasoconstrictors has raised a number of questions about the role of PtdIns turnover in mediating its effects. ET is the most potent vasoconstrictor yet described and produces maximal responses similar to those by other agents, if somewhat slower in onset. Surprisingly, examination of the inositol phosphate and Ca^{2+} responses showed ET to be less effective than VP or AII in terms of the maximum levels of stimulation achieved [30]. Apparently opposite results were obtained in 3T3 fibroblasts, where ET has mitogenic activity. In these cells, the ability of ET to stimulate inositol phosphate accumulation was similar to that observed with another mitogen, bombesin, but the mitogenic response to bombesin was quantitatively much greater than that to ET [17]. Taken together, these findings suggest some unusual properties of the PtdIns-turnover pathway in response to ET and suggest that measuring total inositol phosphates may be a poor reflection of the functional activity of the pathway.

Given the unusual properties of ET-mediated responses, it was decided to address the question of flexibility in the operation of the PtdIns-turnover pathway. In this paper, a detailed examination has been made of the actions of ET on inositol phosphate release and metabolism in adrenal glomerulosa cells, where ET has steroidogenic activity. These profiles have been compared with the well-characterized response to AII, which stimulates aldosterone synthesis via activation of the PtdIns-turnover pathway and associated changes in cytosolic Ca^{2+} [31–33].

Abbreviations used: DAG, diacylglycerol; ET, endothelin; AII, angiotensin II; VP, vasopressin.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparation of rat adrenal glomerulosa cells

Adrenal glomerulosa cells were prepared essentially as described previously [34]. Briefly, adult male Sprague-Dawley rats were killed by decapitation. Adrenals were removed and trimmed of fat. Capsules were dissected, cut up finely with scissors and washed thoroughly in Hepes-buffered medium 199 to remove fat and non-capsular material. Cleaned chopped capsules were incubated with collagenase (2 mg/ml), Dispase (2 mg/ml) and DNAase (50 μ g/ml) for 45 min at 37 °C. Cells were dispersed by gently pipetting up and down in silicone-treated Pasteur pipettes. Dispersed tissue was passed through nylon mesh (80 μ m pore size) and centrifuged at 500 *g* for 5 min. Cells were washed twice with Hepes-buffered medium 199 and resuspended in medium containing 10% (v/v) foetal-calf serum (or 2% BSA) and 100 units each of penicillin and streptomycin/ml.

Generation of inositol phosphates

For inositol phosphate studies, cells were incubated with [3 H]inositol (20 μ Ci/ml) for 24 h to label inositol phospholipids. Non-radioactive inositol was then added and labelled cells were washed twice in medium containing 5 mM-inositol. [3 H]inositol-labelled cells (approx. 2×10^5) were incubated in 0.3 ml of Hepes-buffered medium 199 containing 2% BSA, 5 mM-inositol, 10 mM-LiCl and stimulatory peptide. Incubation was terminated by adding 1 ml of 10% trichloroacetic acid containing 1 mM-EDTA at 0 °C. Samples were sonicated and then left on ice for 10 min before centrifugation. Supernatants were extracted with 4 $\times$ 1 ml of diethyl ether, diluted to 10 ml with water and applied immediately to 1 ml Dowex-1 formate columns. Inositol phosphates were eluted with increasing concentrations of ammonium formate as described previously [35].

The nature of the products separated by Dowex chromatography was validated by anion-exchange h.p.l.c. analysis of the fractions as explained below.

Release of aldosterone

Cells [$(2-5) \times 10^5$] were incubated with peptide for 2 h. Culture medium was removed and stored at -20 °C. Aldosterone measurements were performed by radioimmunoassay using aldosterone dissolved in culture medium as standard.

Estimation of cytosolic free Ca^{2+} concentration

Cells attached to glass cover slips were washed three times in PSS buffer (135 mM-NaCl, 5 mM-KCl, 1.8 mM- CaCl_2 , 0.8 mM- MgSO_4 , 5 mM-glucose and 10 mM-Hepes buffer, pH 7.35) and subsequently incubated in PSS buffer containing 1 μ M fura-2AM for 40 min at 37 °C. Fluorescence was recorded at 505 nm emission with excitation at 340 and 380 nm, with a Spex Fluorolog CM-2 fluorimeter. Fluorescence values (340/380 nm) were calculated after correcting for auto-fluorescence by ionomycin (1 μ M) and MnCl_2 (2 mM). Ca^{2+} concentrations were calculated as described by Grynkiewicz *et al.* [36]. Constants were determined by external calibration of the fluorescence of 20 mM fura-2 free acid.

Separation and determination of the isomers of the inositol phosphates

Where detailed examination of inositol phosphate isomers was required, cells (approx. 5×10^5 /well) were labelled for 24 h with [3 H]inositol (40 μ Ci/ml). Incubation of cells and extraction of trichloroacetic acid-soluble [3 H]inositol-labelled compounds was as described above, except that samples were freeze-dried after ether extraction. Inositol phosphates were separated by anion-exchange h.p.l.c. on a Whatman Partisil 10 SAX column in a

Waters Radial compression unit [37]. The mobile phase involved a gradient of ammonium formate adjusted to pH 3.7 with H_3PO_4 . This involved a linear increase from 0 to 1 M over 45 min, followed by an increase to 2 M over 1 min. The 2 M-formate was continued for a further 30 min. Fractions were quantified by using an on-line β -counting system (Flo-one model CR, from Radiomatic Instruments). This provided integrated peak values. Peaks were identified by comparison with known standards where available. A standard mixture of ATP, ADP and AMP was included with each sample to check retention times.

Materials

myo-[3 H]inositol, [3 H]inositol 1,4,5-trisphosphate, [3 H]inositol 1,3,4,5-tetrakisphosphate and [14 C]inositol 1-monophosphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [3 H]inositol 4-monophosphate was obtained from Du Pont, Wilmington, DE, U.S.A. Medium 199, BSA, penicillin and streptomycin were obtained from the Commonwealth Serum Laboratories (Melbourne, Vic., Australia).

Collagenase (type III) was purchased from Worthington (Freehold, NJ, U.S.A.) and Dispase from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

ET-1 was purchased from Auspep (Melbourne, Vic., Australia). Angiotensin II was Hypertensin (Ciba), and [arginine]-vasopressin was from Peninsular Laboratories (Belmont, CA, U.S.A.).

Aldosterone assay kits were purchased from Biomediq (Melbourne, Vic., Australia).

RESULTS

Steroidogenic activity of ET

AII is an important aldosterone secretagogue *in vivo* and also is effective in stimulating aldosterone synthesis and release from isolated adrenal glomerulosa cells. VP is also able to stimulate production of aldosterone by adrenal glomerulosa cells, but the maximum steroidogenic response was lower than that observed with AII [38]. In these experiments, the effect of ET on aldosterone release by isolated adrenal glomerulosa cells was investigated. Cells were incubated with ET, AII or VP for 2 h. Medium was removed and subsequently assayed for aldosterone. As shown in Fig. 1, increasing concentrations of ET produced a dose-dependent stimulation of aldosterone production, with an average EC_{50} value of 6.3 ± 2.1 nM (mean $\pm$ S.E.M., $n = 6$). The stimulation was small relative to that produced by AII and quantitatively similar to the vasopressin stimulation.

Fig. 1. Aldosterone production by rat adrenal glomerulosa cells: effects of AII, ET and VP

Cells were incubated for 2 h. Medium was removed and assayed for aldosterone. Results shown are means $\pm$ S.E.M. of six experiments.

Fig. 2. Inositol phosphate release in adrenal glomerulosa cells: effects of AII, ET and VP

Cells were labelled with [^3H]inositol and subsequently incubated with peptide in the presence of 10 mM-LiCl for 20 min. Trichloroacetic acid-soluble [^3H]inositol-labelled products were extracted and separated into total InsP_1 , InsP_2 and InsP_3 fractions. Results shown are means $\pm$ S.E.M. of five experiments.

Effect of ET on inositol phosphate accumulation

Cells labelled with [^3H]inositol were incubated with increasing concentrations of ET, AII or VP for 20 min. ^3H -labelled inositol phosphates were extracted and separated into InsP , InsP_2 and InsP_3 fractions. Increasing concentrations of ET produced a dose-dependent stimulation of accumulation of each of the inositol phosphate fractions. The average EC_{50} value was 2.5 ± 0.6 mM (mean $\pm$ S.E.M., $n = 6$). As shown in Fig. 2, the extent of stimulation with ET was not different from that observed with AII. VP produced a smaller maximum stimulation.

Analysis of inositol phosphate metabolism after addition of ET or AII

The apparent discrepancy between the effects of the peptides on steroidogenesis and on total inositol phosphate accumulation suggested some differences in the nature of the pathway. Accordingly, experiments were undertaken to investigate the time course of accumulation of the different inositol phosphate isomers under stimulation by each of the three peptides. Cells were labelled with [^3H]inositol and subsequently incubated with peptide for times between 5 s and 20 min. Trichloroacetic acid-soluble [^3H]inositol-labelled products were separated and quantified by anion-exchange h.p.l.c. as explained in the Materials and methods section.

Addition of ET ($0.1 \mu\text{M}$) caused a rapid and transient increase in $\text{Ins}(1,4,5)\text{P}_3$, which was maximal by 5 s, remained constant until 15 s and had decreased by 1 min. This was followed by a slow, gradual, rise which continued past 20 min. This $\text{Ins}(1,4,5)\text{P}_3$ was rapidly metabolized both via phosphorylation to $\text{Ins}(1,3,4,5)\text{P}_4$ and by dephosphorylation to $\text{Ins}(1,4)\text{P}_2$. Increases in both $\text{Ins}3\text{P}$ (or $\text{Ins}1\text{P}$) and $\text{Ins}4\text{P}$ were detectable as early as 15 s after addition of ET (Fig. 3).

A different profile was observed under stimulation with AII ($0.1 \mu\text{M}$) (Fig. 4). AII caused a rapid, transient, rise in $\text{Ins}(1,4,5)\text{P}_3$, which was maximal by 5 s and had decreased by 15 s (Table 1). As with ET, this was followed by a slow, sustained, rise. The pattern of accumulation of the other inositol phosphates was different from that observed with ET. Early metabolism was primarily via $\text{Ins}(1,4)\text{P}_2$ to $\text{Ins}4\text{P}$, but after 5 min the rates of generation of $\text{Ins}4\text{P}$ and $\text{Ins}3\text{P}$ were parallel, showing that metabolism via the phosphorylation and dephosphorylation pathways were similar. Thus the $\text{Ins}(1,4,5)\text{P}_3$ kinase pathway appears to be more important under prolonged AII stimulation than was observed during the first 1 min after addition of hormone.

Quantitative analysis of the metabolism of $\text{Ins}(1,4,5)\text{P}_3$

To obtain a more quantitative comparison of the pathway, an

Fig. 3. Effects of ET ($0.1 \mu\text{M}$) on the accumulation of inositol phosphates in rat adrenal glomerulosa cells

[^3H]Inositol-labelled cells were incubated with ET for times between 5 s and 20 min. Inositol phosphates were extracted and the isomers separated by h.p.l.c. as described in the Materials and methods section. Note the break in the time axis. The experiment was performed three times with similar results.

estimation was made of the total metabolism of $\text{Ins}(1,4,5)\text{P}_3$ via both pathways. Since the rates of turnover of the various metabolites are unknown, these studies were performed in the presence of LiCl to prevent breakdown of InsP and to inhibit breakdown of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$ [39]. The experiments were similar to those described in Fig. 1 and 2. At each time point total radioactivity (c.p.m.) in $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}4\text{P}$ was calculated as an estimate of the activity of the 5-phosphatase pathway. Similarly, total radioactivity in $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}3\text{P}$ was calculated as an estimate of the activity of the 3-kinase pathway. The ratios of the activities of the two pathways were calculated at each time point in the presence of ET, AII or VP. Results are shown in Fig. 5. Under ET stimulation, the ratio of the activities of the 5-phosphatase and 3-kinase pathways remained constant between 5 s and 20 min. The profile observed under AII stimulation was different. The ratio of 5-phosphatase to 3-kinase activity increased rapidly after addition

Fig 4. Effects of maximally effective concentrations of AII ($0.1 \mu\text{M}$) on the accumulation of inositol phosphates in rat adrenal glomerulosa cells

Cells were labelled with [^3H]inositol and subsequently incubated with AII for times between 5 s and 20 min. Inositol phosphates were extracted and the isomers were separated by h.p.l.c. as described in the Materials and methods section. Note the break in the time axis. The experiment was performed four times with similar results.

Table 1. Effects of ET, AII and VP on the accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in adrenal glomerulosa cells

Results shown are ^3H c.p.m. in $\text{Ins}(1,4,5)\text{P}_3$ after stimulation by ET, AII or VP at the concentrations indicated in the presence of 10 mM-LiCl. Values are means $\pm$ S.E.M. The average zero-time value was 6.2 ± 2.2 ($n = 15$).

Additions	$10^{-3} \times \text{Ins}(1,4,5)\text{P}_3$ (c.p.m./ 10^6 cells)		<i>n</i>
	5 s	15 s	
ET ($0.1 \mu\text{M}$)	15.8 ± 2.6	15.4 ± 2.3	6
AII ($0.1 \mu\text{M}$)	21.2 ± 2.0	12.7 ± 2.3	7
VP ($0.1 \mu\text{M}$)	9.2 ± 1.0	8.0 ± 1.3	5
AII (0.3 nM)	11.3 ± 1.2	6.7 ± 1.3	3
AII ($0.1 \mu\text{M}$) + ET ($0.1 \mu\text{M}$)	24.1 ± 3.1	16.8 ± 2.9	3

of AII, and then decreased between 1 min and 20 min to a value not different from that observed with ET. The profile observed with VP as stimulus also is shown in Fig. 5. In this case the activity of the 5-phosphatase pathway predominated at all time points between 5 s and 20 min.

The observed differences between the responses to the three peptides might have been related either to the overall extent of the stimulation (as shown in Fig. 2) or to the initial rise in $\text{Ins}(1,4,5)\text{P}_3$ (which was higher with AII). Accordingly, experiments were carried out with AII at a concentration (0.3 nM) which produced a small overall InsP response and a small initial rise in $\text{Ins}(1,4,5)\text{P}_3$ (Table 1). The profile of accumulation of the inositol phosphates was qualitatively similar to that observed at maximally effective concentrations of AII and different from those observed with VP and ET. The ratio of 5-phosphatase to 3-kinase activities averaged 2.6 ± 0.38 at 15 s and 1.26 ± 0.48 at 20 min.

Fig. 5. Inositol phosphate metabolism in adrenal glomerulosa cells stimulated with ET, AII and VP

[^3H]Inositol-labelled cells were incubated with peptide in the presence of 10 mM-LiCl for the time indicated. Total radioactivity (c.p.m.) in metabolites of $\text{Ins}(1,4,5)\text{P}_3$ generated via the 5-phosphatase and 3-kinase pathways were calculated as explained in the Results section. Shown is the ratio of activity of the two pathways at times between 5 s and 20 min. Values are means $\pm$ S.E.M. of 3 or 4 experiments. Key: $\circ$, ET ($0.1 \mu\text{M}$); $\blacksquare$, AII ($0.1 \mu\text{M}$); $\diamond$, VP ($0.1 \mu\text{M}$).

Fig. 6. Cytosolic free Ca^{2+} responses in adrenal glomerulosa cells

Cells were loaded with fura-2 AM, and free Ca^{2+} concentrations were determined from the ratio of the fluorescence at 340/380 nm as described in the Materials and methods section. Times of addition of peptide are indicated.

Effects of ET, AII and VP on cytosolic free Ca^{2+} concentrations

For comparison with the inositol phosphate data, experiments were performed to investigate changes in cytosolic free Ca^{2+} concentrations after addition of ET, AII or VP at maximally effective concentrations ($0.1 \mu\text{M}$). Each of the three peptides produced a rapid rise in Ca^{2+} , which quickly decreased to a

sustained value above that observed in unstimulated cells. AII produced the highest response, and responses to VP and ET were similar (Fig. 6).

Additivity of the responses to ET and AII

To ensure that ET and AII were stimulating the same population of cells, experiments were performed in which the additivities of the $\text{Ins}(1,4,5)\text{P}_3$ responses and the Ca^{2+} responses were investigated. When maximally effective concentrations of ET and AII were added together, the observed $\text{Ins}(1,4,5)\text{P}_3$ response at both 5 and 15 s was less than the addition of the two individual responses (Table 1). Similarly, the cytosolic free Ca^{2+} responses to AII and ET were not additive. Addition of AII ($0.1 \mu\text{M}$) increased free Ca^{2+} from $188 \pm 24 \text{ nM}$ (mean $\pm$ S.E.M.; $n = 4$) to 312 ± 25 and 301 ± 8 at 5 s and 15 s respectively. ET increased Ca^{2+} to 285 ± 25 and $296 \pm 13 \text{ nM}$ respectively, and the two peptides together produced an increase to 273 ± 60 and $274 \pm 55 \text{ nM}$. Thus the Ca^{2+} responses to the two peptides were not additive, and the two peptides appear to act on the same population of cells.

DISCUSSION

The present results show that the vasoconstrictor peptide ET acts on adrenal glomerulosa cells to stimulate inositol phosphate generation, to increase cytosolic free Ca^{2+} and to stimulate aldosterone release. This suggests a mechanism of action essentially similar to that of AII and VP in these cells. Both VP and ET initiated steroidogenic and Ca^{2+} responses which were small compared with the response to AII. For VP, these small responses were paralleled by a small stimulation of inositol phosphate accumulation, but ET caused an overall inositol phosphate response which was not different from the AII response. These findings indicated that the relationships between inositol phosphate accumulation, Ca^{2+} and steroidogenesis are not simple and that different responses to different stimuli are possible. Although the stimulation of inositol phosphate release by ET was quantitatively similar to the AII response over a 20 min period, the profile of changes in the different inositol phosphates was different. The ET response differed from that to AII in two ways. First, the initial $\text{Ins}(1,4,5)\text{P}_3$ response was less rapid and the peak height of the initial response was lower. Second, the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ showed a higher percentage flux through the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase pathway, especially at times less than 1 min. The subsequent direction of $\text{Ins}(1,4,5)\text{P}_3$ metabolism did not appear to be related to the initial rate or extent of its release, because inositol phosphate metabolism was different under stimulation by VP and sub-maximal concentrations of AII, even though the initial increases in $\text{Ins}(1,4,5)\text{P}_3$ were similar.

The different profiles of metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in the presence of ET, AII and VP are probably due to modulation of the activities of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase or $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase. The 5-phosphatase is localized partially in the plasma membrane [40] and has been shown to be modulated by protein kinase C in some cells [9]. Thus any difference in the kinetics of DAG release or in the structure of the DAG released might influence $\text{Ins}(1,4,5)\text{P}_3$ metabolism. It is noteworthy that in 3T3 fibroblasts the time course of the DAG response to ET was different from that to bombesin [17], but whether similar differences apply to the adrenal glomerulosa remains to be investigated. The $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase has been shown to be stimulated by Ca^{2+} in several different cell types, including adrenal glomerulosa cells [10–12]. In addition to stimulating PtdIns turnover, AII independently opens Ca^{2+} channels [41,42], and this extra influx of Ca^{2+} may activate $\text{Ins}(1,4,5)\text{P}_3$ kinase. However, Ca^{2+} is unlikely to be the major controlling factor, because the metab-

olism of $\text{Ins}(1,4,5)\text{P}_3$ was different in the presence of VP and ET, even though the Ca^{2+} responses were similar. In addition to PtdIns turnover and Ca^{2+} channels, AII receptors on glomerulosa cells are coupled in an inhibitory manner to adenylate cyclase. Depression of intracellular cyclic AMP [43,44] might influence inositol phosphate metabolism. Effects of cyclic AMP on the overall inositol phosphate response to AII in adrenal glomerulosa cells have been reported previously [38,45], but effects on inositol phosphate metabolism have not been investigated.

The difference between the inositol phosphate profiles observed in the presence of ET and AII might be explained if ET interacted with only a small percentage of the isolated glomerulosa cells, which had an inherently different PtdIns turnover pathway. Although it is not possible to rule out this explanation entirely, it seems unlikely, because the $\text{Ins}(1,4,5)\text{P}_3$ and Ca^{2+} responses to AII and ET were not additive, as would be expected in such circumstances.

In the above discussion it has been assumed that $\text{Ins}(1,3,4,5)\text{P}_4$ and its dephosphorylation products arise from $\text{Ins}(1,4,5)\text{P}_3$. Other possible sources of such products are the newly described inositol lipids phosphorylated at the 3 position: PtdIns3P, PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 [46,47]. Phospholipase C cleavage of these lipids could conceivably generate $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ independently of $\text{Ins}(1,4,5)\text{P}_3$ generation. However, to date synthesis of these lipids has only been described in a limited number of cell types and a limited number of stimuli [47,48]. Furthermore, these 3-phosphorylated lipids appear to be resistant to hydrolysis by phospholipase C [49,50]. Another possible source of $\text{Ins}1\text{P}$ (indistinguishable from $\text{Ins}3\text{P}$) is by direct hydrolysis of PtdIns. It is possible that PtdIns breakdown occurs to different extents with each of the three agonists. However, the accumulation of $\text{Ins}3\text{P}$ under stimulation with the three different agonists paralleled the $\text{Ins}(1,3,4)\text{P}_3$ accumulation, suggesting that most of the $\text{Ins}3\text{P}$ was derived from dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$.

These studies provide further evidence that, even within one cell type, the PtdIns-turnover pathway is not a fixed sequence of events, but can operate differently under different stimulatory conditions. Previous studies have demonstrated differences in the properties of DAG release under stimulation by different effectors, but different inositol phosphate profiles have not been reported previously. Whether such differences in inositol phosphate metabolism are of importance in determining cellular responses, or whether they are merely a reflection of other changes in the cells, remains to be determined. The possibility remains that differences in the nature of the pathway provide an explanation for the different steroidogenic responses to AII, VP and ET.

This work was supported by grants from the Australian National Health and Medical Research Council and the Ramaciotti Foundation. We also acknowledge the technical assistance of Gordon Mill for his help with the Ca^{2+} experiments.

REFERENCES

- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature* (London) **309**, 63–66
- Biden, T. J., Prentki, M., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Biochem. J.* **223**, 467–473
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Shears, S. B. (1989) *Biochem. J.* **260**, 313–324
- Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
- Changya, L., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. (1989) *FEBS Lett.* **251**, 43–48
- Hill, T. D., Dean, N. M. & Boynton, A. L. (1988) *Science* **242**, 1176–1178

8. Joseph, S. K., Hansen, C. A. & Williamson, J. R. (1987) *FEBS Lett.* **219**, 125–129
9. Connolly, T. M., Lawing, W. J., Jr. & Majerus, P. W. (1986) *Cell* **46**, 951–958
10. Biden, T. J. & Wollheim, L. B. (1986) *J. Biol. Chem.* **261**, 11931–11934
11. Rossier, M. F., Capponi, A. M. & Vallotton, M. B. (1987) *Biochem. J.* **245**, 305–307
12. Baukal, A. J., Balla, T., Hunyady, L., Hausdorff, W., Guillemette, G. & Catt, K. J. (1988) *J. Biol. Chem.* **263**, 6087–6092
13. Ambler, S. K., Thompson, B., Solski, P. A., Brown, J. H. & Taylor, P. (1987) *Mol. Pharmacol.* **32**, 376–383
14. Hortsman, D. A., Takemura, H. & Putney, J. W., Jr. (1988) *J. Biol. Chem.* **263**, 15297–15303
15. Peiter-Reish, B., Fathi, M., Schlegel, W. & Wollheim, C. B. (1988) *J. Clin. Invest.* **81**, 1154–1161
16. Pessen, M. S. & Raben, D. M. (1989) *J. Biol. Chem.* **264**, 8729–8738
17. Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K., & Masaki, T. (1989) *J. Biol. Chem.* **264**, 7856–7861
18. Yanagisawa, M., Kurichasa, H., Kumura, S., Tomdei, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) *Nature (London)* **332**, 411–415
19. Moravec, C. S., Reynolds, E. E., Stewart, R. W. & Bond, M. (1989) *Biochem. Biophys. Res. Commun.* **159**, 14–18
20. Hu, J. R., von Harsdorf, R. & Lang, R. W. (1988) *Eur. J. Pharmacol.* **158**, 275–278
21. Hu, J. R., Berminger, V. G. & Lang, R. E. (1988) *Eur. J. Pharmacol.* **158**, 177–178
22. Simonsen, M. S., Wann, S., Meni, P., Dulyak, G. R., Kester, M., Nakazato, Y., Sedon, J. R. & Dunn, M. J. (1989) *J. Clin. Invest.* **83**, 708–712
23. Cozza, E. N., Gomez-Sanchez, C. E., Feeking, M. F. & Chiou, S. (1989) *J. Clin. Invest.* **84**, 1032–1035
24. Morishita, R., Higaki, J. & Ogchara, T. (1989) *Biochem. Biophys. Res. Commun.* **160**, 628–632
25. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T. X., Kumagai, S., Nakajima, K. & Sakakibasa, S. (1988) *Biochem. Biophys. Res. Commun.* **154**, 868–875
26. van Rentorghem, C., Vigni, P., Barhanin, J., Schmidt-Alliana, A., Frelin, C. & Lazdunski, M. (1988) *Biochem. Biophys. Res. Commun.* **157**, 977–985
27. Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A., Jr. & Alexander, R. W. (1986) *J. Biol. Chem.* **261**, 5901–5906
28. Aiyar, N., Nambi, P., Stassen, F. L. & Crooke, S. T. (1986) *Life Sci.* **39**, 37–45
29. Pijuan, V. & Litosch, I. (1988) *Biochem. Biophys. Res. Commun.* **156**, 240–245
30. Wallnofer, A., Weir, S., Rugy, V. & Carvein, C. (1989) *J. Cardiovasc. Pharmacol.* **13**, S23–S31
31. Kojima, I., Lippes, H., Kojima, K. & Rasmussen, H. (1983) *Biochem. Biophys. Res. Commun.* **116**, 555–562
32. Kojima, I., Kojima, K. & Rasmussen, H. (1985) *J. Biol. Chem.* **260**, 9177–9184
33. Capponi, A. M., Lew, P. D. & Vallotton, M. B. (1987) *Biochem. J.* **247**, 335–340
34. Woodcock, E. A., Smith, A. I. & White, L. B. S. (1988) *Endocrinology (Baltimore)* **122**, 1053–1059
35. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–483
36. Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
37. Woodcock, E. A., Smith, A. I., Wallace, C. A. & White, L. B. S. (1987) *Biochem. Biophys. Res. Commun.* **148**, 68–77
38. Woodcock, E. A., McLeod, J. K. & Johnston, C. I. (1986) *Endocrinology (Baltimore)* **118**, 2432–2436
39. Hughes, P. J. & Drummond, A. J. (1987) *Biochem. J.* **248**, 463–470
40. Molina y Vedia, L., Nolan, R. D. & Lapetina, E. G. (1988) *Biochem. J.* **255**, 795–800
41. Aguilera, G. & Catt, K. J. (1986) *Endocrinology (Baltimore)* **118**, 112–118
42. Kojima, I., Shibota, H. & Ogata, E. (1986) *FEBS Lett.* **204**, 347–351
43. Woodcock, E. A. & Johnston, C. I. (1984) *Endocrinology (Baltimore)* **115**, 337–341
44. Marie, J. & Lard, S. (1983) *FEBS Lett.* **159**, 97–101
45. Guillon, G., Gallo-Payet, N., Balestri, M. N. & Lombard, C. (1988) *Biochem. J.* **253**, 765–775
46. Traynor-Kaplan, A. E., Harris, A. L., Thompson, B. L., Taylor, P. & Sklar, L. A. (1989) *Nature (London)* **334**, 353–356
47. Whitman, M., Kaplan, D., Roberts, T. & Cantley, L. (1987) *Biochem. J.* **247**, 165–174
48. Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Omann, G. M. & Klar, L. A. (1989) *J. Biol. Chem.* **264**, 15668–15673
49. Lips, D. L., Majerus, P. W., Gorga, F. R., Young, A. T. & Benjamin, T. L. (1989) *J. Biol. Chem.* **264**, 8759–8763
50. Servinian, L. A., Halus, M. T., Fukui, T., Kim, J. W., Rhee, S. G., Lowenstein, J. M. & Cantley, L. C. (1989) *J. Biol. Chem.* **264**, 17809–17815

Received 23 February 1990/26 June 1990; accepted 16 July 1990