Effects of Angiotensin-II and Potassium on Phospholipid Metabolism in the Adrenal Zona Glomerulosa*

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We examined further the effects of angiotensin-II and K+ on phospholipid metabolism in the adrenal zona glomerulosa. In adrenal zona glomerulosa cell preparations, physiological and supraphysiological concentrations of angiotensin-II (10^-12-10^-4 m) and K+ (0-15 mM) elicited dose- and time-dependent increases in aldosterone production and (32P)phosphate incorporation into chromatographically purified phosphatidic acid and phosphatidylinositol; the stimulatory effect on the phosphatidate-inositol cycle was relatively specific as there were no apparent changes in (32P)phosphate labeled in areas of the chromatogram containing the major phospholipids, phosphatidylcholine and phosphatidylethanolamine. Angiotensin-II also stimulated the turnover of prelabeled (32P)phosphatidylinositol in vitro, and treatment in vivo with a diuretic (provoking Na+ depletion and stimulation of endogenous angiotensin-II secretion) was attended by 2-3-fold increases in phosphatic acid, phosphatidylinositol, and polyphosphoinositides, and lesser increases in other phospholipids. Thus, the stimulatory effect of angiotensin-II on (32P)phosphate labeling observed in vitro may be due to stimulation of two processes, viz. phosphatidylinositol hydrolysis (with a subsequent increase in phosphatidate synthesis) and de novo synthesis of phosphatidic acid.

Cycloheximide inhibited aldosterone production and blocked the angiotensin-II-induced increases in (32P)phosphate incorporation into phosphatidic acid and phosphatidylinositol. Thus, similar to our findings in studies of adrenocorticotropic hormone action in the zona fasciculata-reticularis, a labile protein appears to be required for the stimulation of phospholipid metabolism during angiotensin-II action in the zona glomerulosa.

The present findings provide further evidence to support the possibility that phospholipids play an important role in the stimulation of aldosterone synthesis by angiotensin-II and K+.

Angiotensin-II and K+ are recognized as major regulators of aldosterone biosynthesis and secretion, but little is known concerning their mechanism of action. We have recently provided evidence that adrenocorticotropic hormone controls steroidogenesis via effects on phospholipid metabolism (1-6), and, in a preliminary study (7), angiotensin-II and K+ were found to elicit effects on phospholipid metabolism in the zona glomerulosa which are similar to those evoked by ACTH1 and cAMP in the zona fasciculata-reticularis. However, adrenal capsular preparations were employed in the glomerulosa study, and while this provided enough tissue to permit accurate measurement of phospholipid concentrations, relatively large unphysiological concentrations of angiotensin-II were required to stimulate the capsules. To examine the effects of more physiological concentrations of angiotensin-II and K+, we turned to the highly sensitive adrenal zona glomerulosa cell preparation described by Fakunding et al. (8). As reported here, using this preparation, we have found that physiological concentrations of angiotensin-II and K+ enhance (32P)phosphate incorporation into phosphatidylinositol and phosphatic acid, two major phospholipids in the phosphatidate-inositol pathway. Furthermore, evidence is presented to indicate that this enhanced incorporation reflects the stimulation of two processes, viz. phosphatidylinositol breakdown and enhanced de novo synthesis of phosphatidic acid.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing approximately 200-250 g were kept in environmentally controlled quarters for 1-2 weeks prior to experimental use. Adrenal zona glomerulosa cells were prepared by methods described by Fakunding et al. (8). These cells were resuspended in Medium 199 containing 5 mM K+ and 2 mg of bovine serum albumin/ml, distributed to plastic incubation vials (approximately 200,000-500,000 cells in 0.5 ml of medium), and incubated at 37 °C under 95% O2 and 5% CO2 with 50 μCi of (32P)H3PO4 for 120 min unless indicated otherwise. After incubation, media were separated from cells by centrifugation and stored at -70 °C for subsequent aldosterone analysis. As described previously (1-4), cellular phospholipids were extracted, and phospholipids were purified by thin layer chromatography employing solvent system B. The latter solvent system provides a clean separation of phosphatidylinositol, phosphatic acid, cardiolipin, which in turn migrates just behind cardiolipin, which in turn migrates just behind neutral lipids which are near the solvent front. Radioactive phospholipids were identified by radioautography and acid charring and counted for radioactivity in a liquid scintillation counter as described previously (3).

The effect of endogenously secreted angiotensin-II on adrenal capsular phospholipid concentrations was determined after stimulation of the renin-angiotensin-adrenal axis by acute salt depletion (see Ref. 9) as follows. Rats were treated for 72 h with 10 mg of Depomedrol to suppress endogenous ACTH secretion. Two hours prior to being killed, the rats were injected intraperitoneally with 1 ml of 0.9% saline (controls) or 10 mg of furosemide in 0.9% NaCl. Following decapitation, blood was collected from the severed neck vessels, and adrenals were rapidly removed from carcasses, chilled in ice-cold 0.9% NaCl, and trimmed free of surrounding fat and connective tissue. Adrenal capsules were stripped from the fasciculata-reticularis zone (see Ref. 10).

The abbreviation used is: ACTH, adrenocorticotropic hormone.

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Capsules or decapsulated interior portions from 4 rats were pooled and homogenized and their phospholipids were extracted, purified, and quantified in terms of phosphorus concentration, as described previously (1-4).

Serum and adrenal corticosterone levels were determined by fluoro-rescence in sulfuric acid (11). Serum aldosterone was determined by the method of Gomez-Sanchez et al. (12). Aldosterone in the incubation media was determined by a radioimmunoassay employing $^{32}$P-aldosterone as the labeled ligand as described by Gomez-Sanchez et al. (13).

Medium 199 was obtained from Grand Island Biological Co. Collagenase, type 5, DNase, bovine serum albumin, and cycloheximide were obtained from Sigma. [Val]$^1$angiotensin-II and [Ile]$^1$angiotensin-II were obtained from Sigma and results were similar with both congeners. Carrier-free ($^{32}$P)H$_3$PO$_4$ was obtained from New England Nuclear.

RESULTS

The time course for aldosterone synthesis and ($^{32}$P)phosphate incorporation into glomerulosa phospholipids is shown in Fig. 1. As is apparent, aldosterone increased steadily during the course of incubation, and $10^{-6}$ M angiotensin-II provoked a 2-fold increase therein. Similarly, the rate of ($^{32}$P)phosphate incorporation into phosphatidylinositol remained relatively constant over the course of incubation, and there was a 250% increase provoked by $10^{-6}$ M angiotensin-II. Phosphatidic acid labeling progressed linearly for the first 60 min of incubation and thereafter showed a tendency to level off in angiotensin-II-stimulated glomerulosa cells. However, effects of angiotensin-II were evident at all times of incubation. In contrast to the effects of angiotensin-II on phosphatidylinositol and phosphatidic acid, there were no effects on incorporation of ($^{32}$P)phosphate into the chromatographic area

![FIG. 1. Time course of angiotensin-II action in vitro. Shown here are the effects of $10^{-6}$ M angiotensin (O) versus control (C) on aldosterone synthesis (upper panel) and ($^{32}$P)phosphate incorporation into phosphatidic acid (second panel), phosphatidylinositol (third panel) and the combined chromatographic area (lower panel) containing phosphatidylincholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and sphingomyelin (SM). Mean values (± S.E.) of three determinations are shown.](image1.png)

![FIG. 2. Dose-related effects of angiotensin-II on aldosterone synthesis and ($^{32}$P)phosphate incorporation into designated phospholipids. Incubation time was 120 min. Shown here are mean results (± S.E.) of three determinations.](image2.png)

![FIG. 3. Dose-related effects of K$^+$ on aldosterone synthesis and ($^{32}$P)phosphate incorporation into phosphatidylinositol and phosphatidic acid. Incubation time was 120 min. Shown here are mean results (± S.E.) of three determinations. Other phospholipids were unaffected by K$^+$ (results not shown).](image3.png)
**Angiotensin-II, K⁺, and Glomerulosa Phospholipids**

TABLE I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aldosterone synthesis (ng/10⁶ cells)</th>
<th>(³²P)Phosphate incorporation (dpm/10⁶ cells)</th>
</tr>
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<tbody>
<tr>
<td>None (control)</td>
<td>96 ± 12 (8)</td>
<td>1,040 ± 95 (8)</td>
</tr>
<tr>
<td>Cycloheximide, 0.1 mM</td>
<td>9 ± 2 (8)</td>
<td>1,290 ± 114 (8)</td>
</tr>
<tr>
<td>Angiotensin II, 10⁻⁶ M</td>
<td>218 ± 6 (11*)</td>
<td>2,988 ± 313 (11*)</td>
</tr>
<tr>
<td>Angiotensin II plus cycloheximide</td>
<td>5 ± 1 (8)</td>
<td>1,144 ± 147 (8)</td>
</tr>
</tbody>
</table>

* p versus control = <0.001 (determined by standard t test).

**Fig. 4. Effects of angiotensin-II on phosphatidylinositol hydrolysis.** Adrenal glomerulosa cells were preincubated for 45 min in 5 ml of medium with 1 mCi of (³²P)phosphate in the phospholipid precursor pool. Angiotensin (A-II, 10⁻⁶ M) was then added and the cells were incubated for the times indicated. Results of two experiments have been combined after multiplication by a correction factor to have both zero time values coincident. Mean value (± S.E.) are shown with the number of determinations in parentheses. p was determined by standard t test. Although not shown here, radioactivity in the combined phosphatidylinositol area was nearly constant and not affected by angiotensin-II during the course of the entire incubation.

**Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin.** Thus, acute angiotensin-II treatment in vitro selectively stimulates the phosphatidate-inositol pathway.

Shown in Fig. 2 are the dose-related effects of angiotensin-II on aldosterone synthesis and (³²P)phosphate incorporation into phosphatidic acid, diphosphoinositide, phosphatidylinositol, cardiolipin, and the combined area including phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and sphingomyelin. As is apparent, peak effects on aldosterone and phosphatidylinositol were achieved at 10⁻⁶ M angiotensin-II. Effects of angiotensin-II on phosphatidylinositol were demonstrable at angiotensin-II concentrations as low as 10⁻¹² M. Incorporation of (³²P)phosphate into diphosphoinositide was also stimulated in a number of experiments (e.g. see Fig. 1), but the level of incorporation into this phospholipid was relatively low, and effects of angiotensin-II were not consistently observed. There were no significant effects of angiotensin-II on the labeling of either cardiolipin or the combined area containing phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and sphingomyelin. In all subsequent experiments, we employed angiotensin-II at a dosage of 10⁻⁶ M to provide maximal stimulation.

Potassium also provoked dose-related increases in (³²P)phosphate incorporation into phosphatidic acid and phosphatidylinositol (Fig. 3). These increases correlated reasonably well, although not perfectly, with increases in aldosterone production.

Since cycloheximide (and puromycin) inhibits effects of ACTH on steroidogenesis and phospholipid metabolism (5), it was of interest to test the effects of this inhibitor of protein synthesis on aldosterone synthesis and phospholipid metabolism in the glomerulosa cells. As shown in Table I, 0.1 mM cycloheximide, a dose which inhibits amino acid incorporation by over 90%, did not affect the basal level of (³²P)phosphate incorporation into phosphatidic acid or phosphatidylinositol but completely blocked all stimulatory effects of angiotensin-II thereon. There were no other effects of angiotensin-II or cycloheximide on phospholipids (results not shown). Cycloheximide profoundly inhibited aldosterone production both in control and angiotensin-II-treated glomerulosa cells.

Increased incorporation of (³²P)phosphate into phosphatidic acid and phosphatidylinositol can result from two basic mechanisms, viz. increased phosphatidylinositol breakdown and resynthesis (see Ref. 14), and de novo synthesis of phosphatidic acid and phosphatidylinositol (1-4). The first mechanism, viz. phosphatidylinositol breakdown, was evaluated by following the rate of decrease in labeled phosphatidylinositol during incubation in the presence of absence of angiotensin-II. As shown in Fig. 4, the rate of decrease in labeled phosphatidylinositol was enhanced by the presence of 10⁻⁶ M angiotensin-II, and this suggested that angiotensin-II enhances phosphatidylinositol breakdown. To evaluate the second possibility, viz. de novo synthesis of phosphatidic acid, rats were treated in vivo with furosemide to provoke acute salt loss and stimulation of the renin-angiotensin-adrenal axis in glucocorticoid-suppressed rats. As shown in Table II, basal levels of serum corticosterone were extremely low, both in control and furosemide-treated animals. Also, adrenal corticosterone levels were similar in both groups, indicating that endogenous ACTH secretion was effectively suppressed. Plasma aldosterone levels were markedly increased in the furosemide-treated animals. Capsular concentrations of triphosphoinositide, diphosphoinositide, phosphatidic acid, and phosphatidylinositol increased approximately 2-3-fold in response to furosemide treatment. In addition, phospholipids in the combined phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and sphingomyelin area increased approximately 40% following furosemide treatment. On the other hand, capsular phosphatidylinositol concentration was unchanged, and the concentration of triphosphoinositide, di-
**TABLE II**

*Effects of furosemide treatment in vivo on rat adrenal capsular phospholipids and steroidogenesis*

All rats were treated with 10 mg of Depomedrol intramuscularly 72 h before experimental use to suppress pituitary ACTH secretion. 0.9% NaCl (1 ml) or 0.9% NaCl + furosemide (10 mg) was injected intraperitoneally 2 h before killing by use of a guillotine. Adrenals were rapidly removed from carcasses, chilled, trimmed free of fat and connective tissue, and capsules and interiors (decapsulated adrenals) were prepared as described under “Materials and Methods.” Capsules or interiors from 4 rats were combined, and their phospholipids were extracted, purified, and quantified as described. Serum aldosterone and corticosterone values are nanograms and micrograms/dl, respectively. Tissue concentrations of corticosterone and phospholipids are micrograms and nanograms of phospholipid-phosphorus/100 mg of tissue, respectively. *p* was determined by standard *t* testing.

<table>
<thead>
<tr>
<th>Substance measured</th>
<th>Treatment</th>
<th><em>p</em> versus control</th>
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<tbody>
<tr>
<td>Serum aldosterone</td>
<td>Furosemide</td>
<td></td>
</tr>
<tr>
<td>Serum corticosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal (interior) corticosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular triphosphoinositide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular diphosphoinositide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular phosphatidic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular phosphatidylserine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular phosphatidylinositol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular PC + PE + PG + SM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interior triphosphoinositide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interior diphosphoinositide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interior phosphatidic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interior phosphatidylinositol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interior PC + PE + PG + SM</td>
<td></td>
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</tr>
</tbody>
</table>

| Mean ± S.E.; *n* = 8. | NS¹ | NS² | NS² | NS² | NS² |

¹ NS, not significant or *p* > 0.05.
² PC, PE, PG, and SM, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and sphingomyelin.

phosphoinositide, phosphatidic acid, and phosphatidylserine in the interior portion of the adrenal were unchanged by furosemide treatment. A slight increase (25%) in phosphatidylinositol concentration in the interior portion was observed after furosemide treatment, and this may reflect either contamination of the interior with glomerulosa elements (15) or a low degree of responsiveness of the zona fasciculata-reticularis to angiotensin-II (16).

**DISCUSSION**

The present results demonstrate that physiological concentrations of angiotensin-II and K⁺ enhance incorporation of (32P)phosphate into phospholipids of cells in the zona glomerulosa. The correlation of angiotensin-II-induced increases in aldosterone synthesis and (32P)phosphate labeling of phosphatidic acid and phosphatidylinositol was reasonably good, suggesting that these processes are closely related. Since ACTH-induced increases in phospholipids of the adrenal fasciculata-reticularis may be responsible for stimulatory effects of ACTH on steroidogenesis (1–6), it seems reasonable to suggest that the changes in (32P)phosphate incorporation into glomerulosa phospholipids in response to angiotensin-II and K⁺ may reflect the mechanism whereby aldosterone production is stimulated by these agents. We have observed similar correlations between steroidogenesis and phospholipid metabolism in the action of luteinizing hormone in corpus luteal cells (17), and it is possible that phospholipids may be involved in the action of all steroidogenic agents.

Concomitant inhibition of aldosterone synthesis and phospholipid metabolism by cycloheximide during angiotensin-II action in the zona glomerulosa is of considerable interest in that this inhibitor of protein synthesis (as well as puromycin) concomitantly blocks the effects of ACTH on steroidogenesis and phospholipid metabolism in the zona fasciculata-reticularis. The latter inhibitory effects of cycloheximide are due to inhibition of *de novo* synthesis of phosphatidic acid from glycerol-3'-PO₄ and fatty acyl-CoA, and inhibition of phosphatidic acid synthesis from diglyceride and ATP (18). As discussed below, angiotensin-II appears to increase phosphatidate synthesis via both mechanisms. Concomitant inhibition of angiotensin-II-induced increases in aldosterone production by cycloheximide is in keeping with the possibility that changes in phospholipid metabolism are important in the stimulation of aldosterone synthesis by angiotensin-II. However, the fact that the control rate of aldosterone synthesis was also inhibited by cycloheximide is disconcerting, as this was not the case in studies of the adrenal fasciculata-reticularis (1–6). Conceivably, cycloheximide may inhibit aldosterone synthesis by direct effects on steroidogenic enzymes or, alternatively, the “basal” rate of steroidogenesis in the present incubation system may be dependent upon protein synthesis and phospholipid metabolism which is not reflected by (32P)phosphate labeling.

Prior to the demonstration that ACTH (1–6, 19), parathyroid hormone (20), angiotensin-II, and K⁺ (7) provide sizeable increases in the levels of phosphatidic acid, phosphatidylinositol, and polyphosphoinositides in their target tissues, the only known mechanism for enhanced incorporation of (32P)phosphate into phospholipids of the phosphatidate-inositol cycle was via phospholipase-C-mediated phosphatidylinositol hydrolysis and resynthesis, as described originally by Hokin and Hokin (21) and extensively reviewed by Michell (14). In studies of ACTH action in the adrenal fasciculata-reticularis, Schrey and Rubin have reported (22) and we have confirmed² that ACTH does not decrease the levels of prelabeled (with inositol) phosphatidylinositol, and phospholipase-C-mediated hydrolysis therefore seems unlikely in ACTH action. Thus, aside from reported deacylation-reacylation (22), the only apparent effect of ACTH on phosphatidylinositol metabolism is to enhance its synthesis, presumably via *de novo* synthesis of phosphatidic acid (1–6, 19). As shown presently, it appears that angiotensin-II influences phosphatidylinositol by at least two mechanisms in the zona glomerulosa. Enhanced breakdown of phosphatidylinositol was clearly observed in *in vitro* studies, and apparently similar enhancement of phosphatidylinositol breakdown (via phospholipase-Ç)² has been shown to occur in hepatocytes during treatment with angiotensin-II (23). In addition, the *present in vivo* experiments clearly indicate that phosphatidylinositol levels are markedly increased by endogenously secreted angiotensin-II, and it therefore seems likely that angiotensin-II enhances *de novo* synthesis of phosphatidic acid and phosphatidylinositol and its derivatives, as well as the degradation of phosphatidylinositol. The combination of both effects, i.e. enhanced

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synthesis and degradation, underscores the profound degree of enhancement of phospholipid turnover during angiotensin-II stimulation.

The relatively small amounts of adrenal glomerulosa tissue after purification by the collag enase dispersion method precluded the possibility of directly measuring changes in concentrations of phospholipids in the in vitro incubation system. However, increases in all phospholipids after furosemide treatment in vivo suggest increased de novo synthesis of capsular phospholipids, and, presumably, these changes induced by diuretic administration in the intact rat were due to angiotensin-II. The latter reasoning is supported by the fact that there was no evidence for an increase in ACTH secretion (cortisol metabolism) (23, 25, 26).

ACTH and parathyroid hormone, appeared to elicit this response via cAMP (2, 3, 24). Angiotensin-II, however, is thought to employ Ca²⁺ as a "second messenger" rather than cAMP (23, 25, 26). Thus, the de novo phospholipid response may not be unique in the action of agents which operate via cAMP. Whether either of the two presently observed effects of angiotensin on adrenal phospholipid metabolism precede or follow changes in Ca²⁺ remains for future investigation.

REFERENCES