Aspects of Angiotensin Action in the Adrenal

**Key Roles for Calcium and Phosphatidyl Inositol**

**MARY E. ELLIOTT, A.B., ROBERT C. ALEXANDER, B.S., AND THEODORE L. GOODFRIEND, M.D.**

**SUMMARY**

The steps between exposure of bovine adrenal glomerulosa cells to angiotensin and the stimulated increase in aldosterone production were studied in two ways. Binding of angiotensin to receptors, and hormone effects on phosphatidyl inositol turnover, \( ^{45} \text{Ca}^{+} \) fluxes, and aldosterone production were measured directly. Other potential intermediate steps were investigated indirectly by use of inhibitors. Angiotensin slowed calcium influx and accelerated phosphatidyl inositol turnover in proportion to hormone dose. The effects correlated with receptor binding and aldosterone production. None of the inhibitors tested, except saralasin, inhibited angiotensin’s effect on phosphatidyl inositol turnover. Altered calcium flux and stimulated aldosterone production were affected by the calmodulin inhibitor trifluoperazine and the intracellular calcium antagonist 8-(N,N-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8).

Several reagents did not affect angiotensin binding, its effect on phosphatidyl inositol, or \( ^{45} \text{Ca}^{+} \) flux, but severely inhibited steroidogenesis. These included the phospholipase A, inhibitor mepacrine, the protein synthesis inhibitor cycloheximide, and the Na+/K-ATPase inhibitor ouabain. Colchicine had very little effect on the processes we measured, suggesting that microtubules play no role in angiotensin action in the adrenal. Based on these observations, we propose that angiotensin II affects the adrenal glomerulosa cell by first interacting with receptors, then increasing phosphatidyl inositol turnover, then altering cellular calcium distribution. Steps distal to altered calcium distribution that contribute to increased steroid output include altered phospholipid metabolism, protein synthesis, and Na/K metabolism.

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**KEY WORDS**

- aldosterone
- calmodulin
- phospholipase
- phospholipid
- adrenal glomerulosa
- protein synthesis
- potassium
- Na/K ATPase

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**ANGIOTENSIN** stimulates aldosterone synthesis by the adrenal glomerulosa. The peptide binds to receptors on the plasma membrane, and by an unknown intermediary mechanism increases the rate of cholesterol side-chain cleavage and corticosterone hydroxylation.

There are several second messengers and processes commonly invoked to explain the mechanism of action of peptide hormones. These include cyclic nucleotides, calcium, other cations, phospholipid metabolism, arachidonic acid metabolism, protein synthesis, and protein phosphorylation. In the case of angiotensin, there is little evidence of a role for cyclic nucleotidies, but considerable data favoring participation by calcium, potassium, phospholipid, and arachidonic acid metabolism, protein synthesis, and protein phosphorylation.

We have studied the mechanism of angiotensin action in adrenal glomerulosa cells by direct measurements of some processes and inhibition of others. Receptor binding, calcium fluxes, phosphatidyl inositol turnover, and aldosterone synthesis were measured directly. Calcium distribution, phospholipase, protein synthesis, microtubules, and Na/K ATPase were inhibited by specific agents.

**Materials and Methods**

**Reagents**

Collagenase I (clostridium histolyticum collagenase EC 3.4.24.3) was obtained from Worthington (Freehold, New Jersey), and bovine serum albumin, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), mepacrine, cardiolipin, and L-α-dipalmitoyl lecithin were obtained from Sigma (St. Louis, Missouri). Phosphatidyl inositol and phosphatidyl ethanolamine were obtained from Supelco (Bellefonte, Pennsylvania). Colchicine was obtained from...
Fisher and ouabain was purchased from Aldrich (Milwaukee, Wisconsin). Angiotensin II (ile8) and sar4-ala8-angiotensin II were obtained from Bachem Company (Torrance, California). The 8-(N,N-diethylamino)-octyl 3,4,5-trimethoxy benzoate hydrochloride (TMB-8) was a gift from Dr. R. J. Smith of the Upjohn Company, nifedipine was supplied by Dr. Garret Gross (Medical College of Wisconsin), and trifluoperazine was a gift from Dr. Frank Siegel, University of Wisconsin. Thin layer chromatography plates were obtained from Analabs (North Haven, Connecticut). Polyethylene tubes for aldosterone radioimmunoassay were purchased from the Sarstedt Company (Princeton, New Jersey). Microcentrifuge tubes were obtained from Stockwell Scientific Corporation (Monterey, California), and 45Ca++ (4-30 Ci/g) and 32P04 (285 Ci/mg) were obtained from New England Nuclear. The [11B] aldosterone and aldosterone antibody were obtained from Diagnostic Products International (Elk Grove Village, Illinois). All reagents and products International were purchased from Research Products International (Elk Grove Village, Illinois). All other compounds were reagent grade and obtained commercially.

Cell Preparation

Adrenal glands from adult cows were obtained from Oscar Mayer Company. Cells were prepared by collagenase digestion of the outer slice of several glands according to a previously published method, except that 3.6 mM KCl was included in all buffers, collagenase was used at 4 mg/ml, and no DNase was used.

Immediately before use, an aliquot of cells, either freshly prepared or after 1 to 2 days storage at 4° C, was filtered through one layer of cheesecloth and centrifuged for 15 minutes at 100 x g. The supernatant was decanted, and cells were resuspended in 5 ml HEPES buffer, NaCl 136.9 mM; MgSO4 1.0 mM; CaCl2 0.5 mM; glucose 11 mM, HEPES 20 mM; 0.1% bovine serum albumin, pH 7.4. Resuspended cells were purified by one or more centrifugations at 100 x g for 2 minutes in 50 ml of buffer. Fasciculata cells could be differentiated from glomerulsa cells by their larger size. With this method, contamination by fasciculata was less than 5% in all preparations.

45Ca++ Influx

These experiments were performed as previously reported, except that the HEPES buffer described above was used for all incubations. Cells and 45Ca++ were incubated in the presence or absence of inhibitors and angiotensin. Inhibitors were added in buffer, with the exception of nifedipine which was added in 0.06 ml of 1% ethanol. The final volume was 0.3 ml. Tubes were incubated and aliquots were taken as previously described. Results are expressed in terms of 45Ca++ taken up by the cells during the first 10 minutes of incubation.

Binding Experiments with Labeled Angiotensin

Cells (800,000), inhibitors, and 11B-angiotensin II (0.03 μCi) in HEPES buffer were incubated for 30 minutes at 37° C in 1.5 ml microcentrifuge tubes. The
final incubation volume was 1.0 ml. Control tubes contained 5 μg of unlabeled angiotensin II. Incubations were terminated by centrifugation for 1 minute in a Beckman Microfuge B. The sides of the tubes and surface of the pellets were rinsed with 1 ml HEPES buffer gently added and aspirated. The cell pellets were counted in a gamma scintillation counter.

Results

"Ca" Influx Experiments

Figure 1 illustrates the effect of angiotensin on "Ca" influx into adrenal glomerulosa cells. Figure 2 illustrates binding of [125I]-angiotensin and the hormone's effects on steroid production in glomerulosa cells. Figure 3 shows a dose-response curve for angiotensin inhibition of "Ca" influx into cells. There is a close correlation between the dose-response curves for angiotensin effects on calcium and aldosterone.

If cytosolic calcium is assumed to be 10^{-7} M, over 90% of the "Ca" taken into the cells was bound or concentrated in subcellular organelles. Furthermore, "Ca" influx is slowed by oligomycin, dinitrophenol, or caffeine, agents that can release calcium bound to organelles (data not shown). Therefore, the effect of angiotensin on exchangeable cell calcium we observed may reflect an effect on intracellular calcium distribution.

Phosphatidyl Inositol Turnover

Figure 4 shows a dose-response curve for stimulation of PO_4 incorporation into phosphatidyl inositol by angiotensin II. As in the case of calcium flux, there was a good correlation between that biochemical event and the stimulation of aldosterone production. Incorporation into other phospholipids was unaffected by angiotensin.

Inhibitors

Saralasin (sar'-ala'-angiotensin II), a competitive inhibitor of angiotensin II, blocked binding of labeled angiotensin and the hormone's effects on calcium flux, phosphatidyl inositol turnover, and aldosterone production (table 1).

Figure 5 shows that trifluoperazine, a calmodulin inhibitor, blocked angiotensin's effects on "Ca" fluxes and aldosterone synthesis to the same degree.
TMB-8, an inhibitor of the actions of intracellular calcium, had similar effects. Calmodulin is known to participate in many intracellular processes, including calcium movements, and these results implicate calmodulin and intracellular calcium in angiotensin-stimulated aldosterone production. Neither reagent inhibited hormone binding nor the stimulation of phosphatidyl inositol turnover by angiotensin (Table 1). These results suggest that calmodulin and intracellular calcium act downstream from receptor binding and the hormone's phosphatidyl inositol effect.

Figure 6 shows that the protein synthesis inhibitor cycloheximide blocked angiotensin-stimulated aldosterone production, but had no effect on 
Ca²⁺ fluxes. The phospholipase A₂ inhibitor mepacrine gave similar results. Neither of these reagents affected phosphatidyl inositol turnover or ³²PO₄²⁻ incorporation (Table 1). Ouabain, a Na⁺/K⁺-ATPase inhibitor, also inhibited aldosterone production without affecting 
Ca²⁺ fluxes. Ouabain did not inhibit angiotensin II binding to cells, and had only a small inhibitory effect on phosphatidyl inositol turnover at 10 μM (Table 1).

### Table 1. Effects of Inhibitors on Angiotensin Binding, Phosphatidyl Inositol Turnover and Aldosterone Synthesis

| Reagent (μM) | Binding of 
| binding (125I-AII (% of control)) | PI turnover (% of All-stimulated ³²PO₄²⁻ incorporation) | Aldosterone synthesis (% of All-increased steroid) |
|--------------|---------------------------------|-------------------------------------------------|---------------------------------------------|
| Control      | 100                             | 100                                             | 100                                         |
| Saralasin (5) | 0                               | 22                                              | 5                                           |
| TMB-8 (100)  | 159                             | 87                                              | 30                                          |
| Trihexoperazine (30) | 115                        | 119                                             | 36                                          |
| Cycloheximide (10) | 94                          | 82                                              | 9                                           |
| Mepacrine (100) | 114                         | 122                                             | 0                                           |
| Ouabain (10) | 92                              | 69                                              | 3                                           |
| Nifedipine (10) | 90                           | 103                                             | 67                                          |
| Colchicine (50) | 103                         | 141                                             | 83                                          |
TRIFLUOPERAZINE

**FIGURE 5.** Effect of trifluoperazine (calmodulin inhibitor) on aldosterone synthesis and $^{45}$Ca$^{+}$ influx in the presence and absence of $10^{-4}$ M angiotensin II. The black bars in each group represent basal (−) and hormone-stimulated (+) steroidogenesis at a given drug concentration, and the white bars represent $^{45}$Ca$^{+}$ influx during the first 10 minutes of exposure to $^{45}$Ca$^{+}$ in the absence (−) and presence (+) of angiotensin. Each set of four bars shows results with one drug concentration. Each $^{45}$Ca$^{+}$ incubation tube contained $2.5 \times 10^6$ cells and $1 \times 10^8$ cpm $^{45}$Ca$^{+}$.

Figure 7 illustrates the effect of nifedipine, a compound known to inhibit the slow calcium channel in heart muscle. This reagent at $10 \mu M$ slowed $^{45}$Ca$^{+}$ uptake into adrenal cells so that no additional effect of angiotensin was seen, although steroid synthesis was still partially stimulated. Angiotensin binding to cells and stimulation of phosphatidyl inositol turnover were unaffected (table 1). It is possible that angiotensin II releases bound intracellular calcium stores in the presence of nifedipine, but the drug masks our ability to measure effects of angiotensin II on $^{45}$Ca$^{+}$ influx. More important, this result shows that, even when a channel blocker prevents calcium entry into cells, angiotensin II can still stimulate steroidogenesis. If calcium is necessary for angiotensin II action, therefore, it must be calcium which is already present in the cell.

Colchicine and other antimicrotubule agents have been reported to inhibit ACTH stimulation of corticosterone synthesis. Colchicine (50 μM) had little effect on angiotensin binding, calcium flux, phospholipid turnover, or aldosterone production.

**Discussion**

Our results with calcium influx and phosphatidyl inositol turnover show that these events are closely linked to angiotensin stimulation of aldosterone production, as witnessed by their rapid change after exposure of adrenal cells to the hormone, and the close correlation between dose-response curves for the three processes. Results published recently by Farese et al. confirm the effect of angiotensin on phospholipid metabolism. Measuring these events does not permit us to assign a sequence to them, however, nor to indicate other processes that might be important. We used inhibitors of various biochemical processes for that purpose, and all of our conclusions depend on the specificity of the reagents we employed. Table 2 summarizes our results. Saralasin, a competitive inhibitor of angiotensin II receptors, blocked angiotensin binding to cells and the hormone's effects on phosphatidyl inositol turnover, $^{45}$Ca$^{+}$ flux, and aldosterone synthesis. No inhibitor specifically blocked phospholipid turnover, but some inhibitors that did not block hor-
mone binding or phospholipid turnover did block calcium flux and steroidogenesis. These were TMB-8, an antagonist of the action of intracellular calcium, and trifluoperazine, a calmodulin inhibitor. Based on these results, we conclude that calmodulin and calcium fluxes play a role in stimulating aldosterone synthesis, but at a point downstream from receptor binding and phosphatidylinositol turnover.

Results with inhibitors of phospholipase A₂ (mepacrine), protein synthesis (cycloheximide), and Na⁺/K⁺-ATPase (ouabain) show pronounced blockade of aldosterone production at drug concentrations that have little or no effect on angiotensin receptors, phosphatidylinositol turnover, or calcium flux. These results suggest roles for protein synthesis, phospholipase A₂, and intracellular potassium levels in aldosterone synthesis downstream from angiotensin receptors, phospholipid turnover, and calcium flux.

Our experiments shed no light on the possible participation of arachidonic acid metabolism, protein phosphorylation, or specific enzyme activation in the stimulation of aldosteroneogenesis. Measurement of these processes and the effects of inhibitors on them should provide further information about the mechanism of action of angiotensin in its target cells.

### Acknowledgment

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### References


### Table 2. Inhibition of Angiotensin's Effects on Adrenal Glomerulosa Cells by Various Agents

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Site of inhibition</th>
<th>Effect on measured event</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AII binding</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Microtubules</td>
<td>0</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Protein synthesis</td>
<td>0</td>
</tr>
<tr>
<td>Mepacrine</td>
<td>Phospholipase</td>
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</tr>
<tr>
<td>Ouabain</td>
<td>Na⁺/K⁺-ATPase</td>
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<tr>
<td>Trifluoperazine</td>
<td>Calmodulin</td>
<td>0</td>
</tr>
<tr>
<td>TMB-8</td>
<td>Intracellular calcium</td>
<td>0</td>
</tr>
<tr>
<td>Saralasin</td>
<td>All receptor</td>
<td>X</td>
</tr>
</tbody>
</table>

0 = no effect; X = inhibition.
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