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Angiotensin alters $^{45}Ca^{2+}$ fluxes in bovine adrenal glomerulosa cells

(aldosterone/steroidogenesis/cation flux/polypeptide hormones/receptors)

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ABSTRACT Angiotensin II stimulated $^{45}Ca^{2+}$ release from bovine adrenal glomerulosa cells. It also decreased the influx of $^{45}Ca^{2+}$ into glomerulosa cells. The effects were observed within 2 min of hormone addition and were blocked by Saralasin, a competitive inhibitor of angiotensin. Des-Phe^{8}-angiotensin II, a biologically inert analog, was inactive in this system. Angiotensin II also inhibited the influx of $^{133}Ba^{2+}$ and $^{54}Mn^{2+}$, whereas $^{51}Cr^{6+}$ and $^{57}Co^{2+}$ were unaffected. Alterations in $^{45}Ca^{2+}$ fluxes were seen with concentrations of angiotensin that stimulate aldosterone biosynthesis in bovine glomerulosa cell preparations. These results suggest that calcium plays a key role in angiotensin-stimulated aldosteronogenesis.

Angiotensin II stimulates aldosterone synthesis both *in vivo* and *in vitro* (1–8). It binds to specific receptors that are probably localized on the plasma membrane of adrenal glomerulosa cells (9, 10). Recent work has shown that angiotensin II increases the activity of enzymes catalyzing side-chain cleavage of cholesterol and 18-hydroxylation of corticosterone (11, 12). However, the mechanism by which angiotensin II-receptor interaction leads to these effects is poorly understood. Cyclic AMP apparently does not act as the second messenger in this system (8, 13–15), and although potassium is required for steroidogenesis, there is no convincing evidence that this ion is the second messenger for angiotensin II (16–19). Prostaglandins may augment or modulate effects of angiotensin II in the adrenal gland, but there is no direct proof that they are fundamentally responsible for its stimulation of steroidogenesis (20–24).

Calcium apparently plays an important role in many of the actions of angiotensin II (13). Calcium in the medium is necessary for the hormone's effects on contraction of rat descending colon (25), for its stimulation of hepatic gluconeogenesis from some substrates (26), and for aldosterone synthesis (8, 14, 27, 28). Lanthanum and other calcium antagonists have been shown to inhibit angiotensin II-stimulated steroidogenesis (8, 14, 29). Angiotensin II released intracellular calcium in rabbit aorta (30). It stimulated glycogenolysis in a cyclic AMP-independent, calcium-dependent fashion (31–34). Garrison *et al.* (34) demonstrated that the effects of angiotensin II and α-adrenergic agonists on the phosphorylation of hepatic glycogenolytic and gluconeogenic enzymes were calcium-dependent. Work from Exton's laboratory (35) has demonstrated that angiotensin II and α-adrenergic agonists alter calcium fluxes in liver. The calcium ionophore A23187 stimulates a modest rise in aldosterone synthesis by adrenal glomerulosa cells (29).

Based on the above connections between angiotensin II and calcium, we sought evidence for a role of calcium as an intracellular mediator of angiotensin II stimulation of aldosterone synthesis. We measured the effects of angiotensin II on calcium fluxes and steroidogenesis in bovine adrenal glomerulosa cells. Our initial results showed a rapid effect of angiotensin II on calcium flux (36), and these observations are described.

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MATERIALS AND METHODS

Tissue and Reagents. Bovine adrenal glands were obtained from the Oscar Mayer Company (Madison, WI). Collagenase I (*Clostridium histolyticum* collagenase EC 3.4.24.3) was obtained from Worthington, and DNase (deoxyribonucleate 5'-oligonucleotidohydrolase; EC 3.1.21.1) and bovine serum albumin were obtained from Sigma. $^{45}Ca^{2+}$ (4–30 Ci/g; 1 Ci = 3.7×10^{10} becquerels), $^{133}Ba^{2+}$ (7.8 mCi/mg), $^{54}Mn^{2+}$ (7.7 Ci/mg), $^{57}Co^{2+}$ (8.5 Ci/mg), $^{51}Cr^{6+}$ (200 mCi/mg), and [^{3}H]aldosterone (140 mCi/mg) were obtained from New England Nuclear. Peptides were obtained from Bachem Company (Torrance, CA). Microcentrifuge tubes were from Stockwell Scientific Corporation (Monterey, CA). Bray's scintillation fluid and polyethylene minivials for scintillation counting were from Research Products International (Elk Grove Village, IL). Aldosterone antibody was a gift of the NIH Hormone Distribution Committee (Bethesda, MD). All other compounds were reagent grade and obtained commercially.

Cell Preparation. Bovine adrenals were obtained at the slaughterhouse, freed of most of the surrounding tissue, and immersed in cold Krebs–Ringer buffer within 30 min of killing. This buffer contained 115 mM NaCl/10 mM Na phosphate/1 mM $MgSO_4$/22 mM $NaHCO_3$/1.5 mM $CaCl_2$/11 mM glucose (pH 7.4). In the laboratory, each adrenal was prepared by peeling off the capsule, bisecting the gland, and scraping away the medulla. Tissue was cut into squares 1 cm wide, and one 0.5-mm slice was cut from the outer surface of each square with a Stadie–Riggs microtome. All slices were cut into smaller pieces and placed into tubes (nitrocellulose, 3.8 × 10.2 cm) containing Krebs–Ringer buffer with 1% crystalline albumin, 7.4% (wt/vol) collagenase, and 0.1% DNase. Of this enzyme solution, 40 ml were used for slices from eight adrenals. Tissue was incubated in a 37°C shaking water bath (60 cycles/min) under a stream of 95% O_2/5% CO_2 for 60 min, and the contents of each tube were mixed at 15-min intervals by pipetting up and down 25 times through a wide-mouth 10-ml pipette.

At the end of 60 min, the digested tissue was filtered through one layer of cheesecloth, and the filtrate was divided into two tubes and centrifuged at 100 × *g* for 15 min in a refrigerated International model PR-2 centrifuge. The supernatant was aspirated, and each cell pellet was resuspended in 15 ml of Krebs–Ringer buffer, which contained 1% crystalline albumin and 3.6 mM KCl. The suspension was incubated for 60 min at 37°C in a shaking water bath under a stream of 95% O_2/5% CO_2. Tubes again were centrifuged at 100 × *g* for 15 min, the supernatant was aspirated, and the entire cell preparation was resuspended in 50 ml of Krebs buffer with 0.1% crystalline albumin and 3.6 mM KCl. Cells were counted in a hemocytometer, and cell viability was monitored by observing exclusion of trypan blue. The yield from eight adrenals was 200–400 × 10^{6}

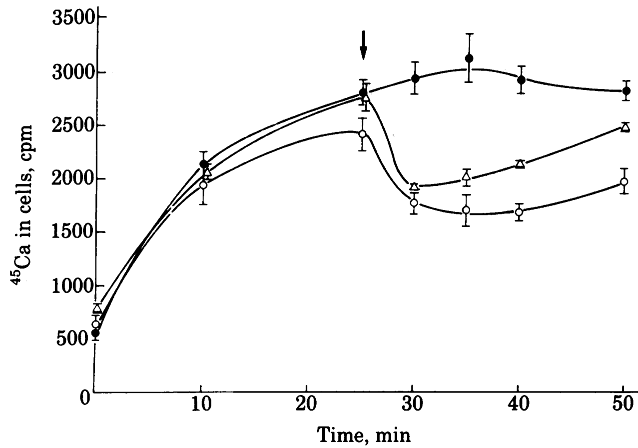

FIG. 1. Effect of angiotensin II on $^{45}Ca^{2+}$ content of bovine adrenal glomerulosa cells. Hormone was added at the arrow. ●, Control; ○, 0.1 μM angiotensin II; △, 0.01 μM angiotensin II.

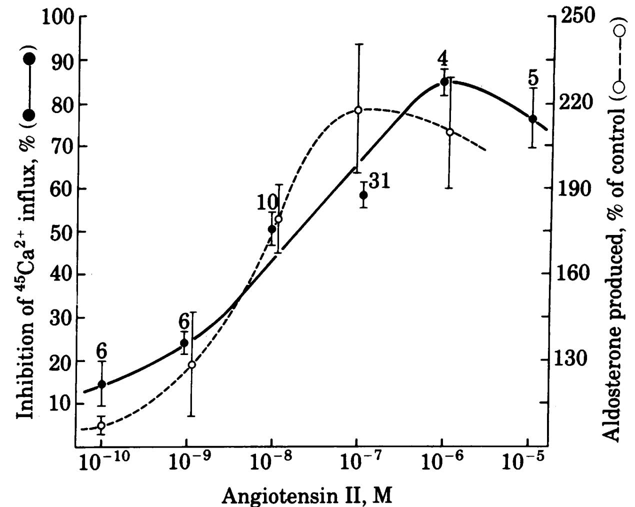

FIG. 3. Dose–response curves for angiotensin II effects on $^{45}Ca^{2+}$ influx and aldosteronogenesis in bovine adrenal glomerulosa cells. Each point on the solid line represents the percentage inhibition of $^{45}Ca^{2+}$ influx during the first 10 min relative to influx with no hormone. The number of experiments averaged for each point is noted above the standard error bars. The dose–response relationship for aldosterone synthesis was obtained from three separate experiments in which cells were incubated with hormone for 2 hr.

cells. Cells were responsive to angiotensin when stored at 4°C for at least 2 days. Aldosterone synthesis was increased 2- to 5-fold by angiotensin II concentrations above 0.1 nM.

Cation Influx Experiments. An aliquot of cells, either freshly prepared or after 1–2 days of storage at 4°C, was centrifuged for 15 min at 100 × g. The supernatant was decanted, and cells were resuspended in either 10 ml of Krebs buffer with 1% crystalline albumin and 3.6 mM K^+ with no Ca^{2+}, or in a modified Krebs buffer (Krebs/Hepes). The modified Krebs buffer (pH 7.4) contained no phosphate or bicarbonate but did contain 10 mM Hepes and varying amounts of $CaCl_2$. When this buffer was used, incubations were carried out in ambient air. Cells were recentrifuged and the supernatant was decanted. Cells were resuspended in the same buffer at a concentration of 5–10 × 10^6 cells per ml.

Techniques for measuring $^{45}Ca^{2+}$ fluxes were based on those used in Lardy's laboratory, where $^{45}Ca^{2+}$ fluxes in liver cells were measured (37). Cells were pipetted (0.3–0.6 ml per tube) into nitrocellulose tubes (1.5 × 7.5 cm) on ice.

Varying concentrations of angiotensin II (0.01 ml) were added, approximately 1 μCi of $^{45}Ca^{2+}$ or other radionuclide was added (0.03 ml), and the tube contents were mixed. To determine cell-associated radioactivity, aliquots of 0.05 ml were withdrawn from each tube and gently placed on top of 0.35 ml of layering solution in 0.4-ml microcentrifuge tubes. The layering solution consisted of 7% (wt/vol) fraction V bovine serum albumin with 0.9% NaCl and 5 mM $CaCl_2$. Tubes were centrifuged for 1 min at 10,000 × g in a Beckman Microfuge B. Supernatants were aspirated, tubes were gently filled with layering solution to rinse away extraneous radioactivity, and the rinse was aspirated.

After zero-time aliquots were taken, the incubation tubes were placed in a 37°C shaking water bath. Aliquots were withdrawn at various times and treated as described. Supernatants were aspirated, and 0.35 ml of 1% $NaDodSO_4$ was added to each cell pellet. After the cell pellets were allowed to dissolve overnight, they were transferred to minivials, and the tubes were washed with an additional 0.35 ml of $NaDodSO_4$. Bray's scin-

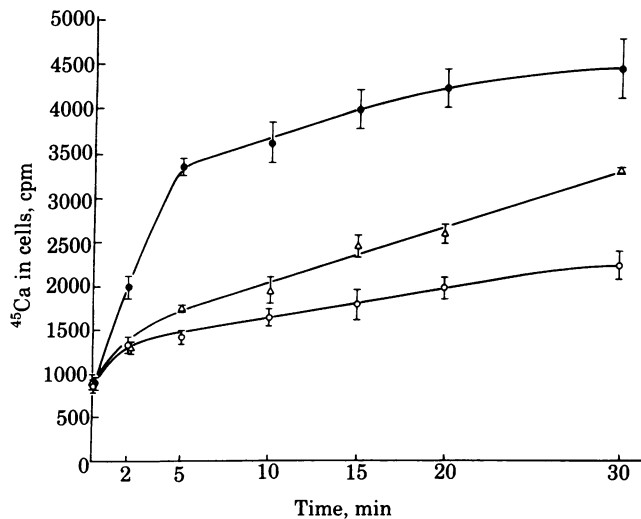

FIG. 2. Effect of angiotensin II on uptake of $^{45}Ca^{2+}$ by glomerulosa cells. Hormone and $^{45}Ca^{2+}$ were added at time zero. ●, Control; ○, 0.1 μM angiotensin II; △, 0.01 M angiotensin II.

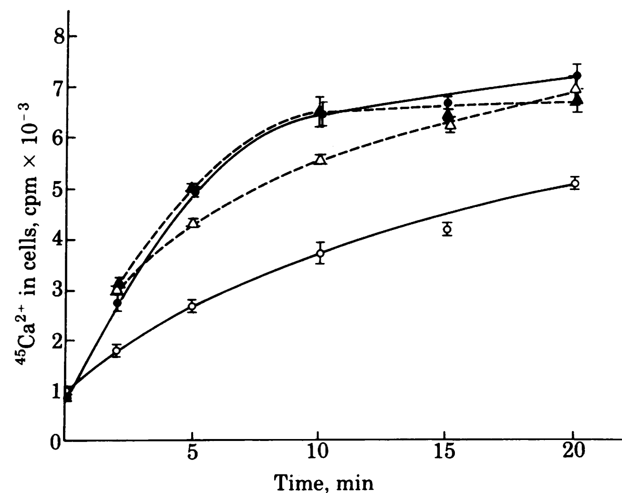

FIG. 4. Effect of angiotensin II, Saralasin, and both on uptake of $^{45}Ca^{2+}$ by glomerulosa cells. Control cells (●——●) were incubated with no hormone. ○——○, Angiotensin II (0.01 μM); ▲- - -▲, Saralasin (0.5 μM); △- - -△, Saralasin and angiotensin II.

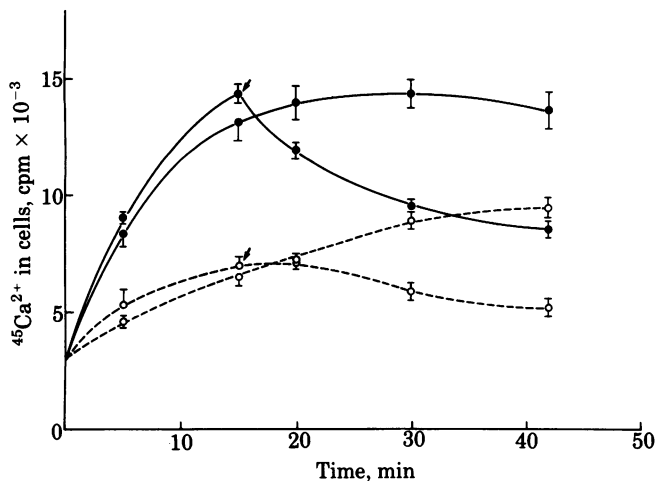

FIG. 5. Effect of La^{3+} addition on angiotensin II alteration of $^{45}Ca^{2+}$ fluxes. At time zero, all incubation tubes received $^{45}Ca^{2+}$ and either buffer (●——●) or 0.1 μM angiotensin II (○---○). After a 15-min incubation, La^{3+} (final concentration, 2.5 mM) was added to tubes indicated by arrows.

tillation solution (3.0 ml) was added to vials, and the vials were counted in a Packard liquid scintillation spectrometer. Aliquots of suspended cells were removed from incubation tubes, and radioactivity was assayed in a solution containing 0.70 ml of 1% NaDodSO$_4$ and 3.0 ml of Bray's solution to give an estimate of the total radioactivity before centrifugation. Radionuclides other than $^{45}Ca^{2+}$ emitted gamma rays and were measured in a well-type counter.

$^{45}Ca^{2+}$ Efflux Experiments. These experiments were similar to the influx experiments, except that cells were incubated with $^{45}Ca^{2+}$ at 37°C for 25 min before the addition of angiotensin II. Aliquots were withdrawn at various times before and after angiotensin II was added.

Aldosterone Synthesis. Cells were tested for aldosterone production in the following manner. After preincubation and centrifugation, cells were resuspended in Krebs-Ringer buffer with 3.6 mM KCl to give a final concentration of 3-5 × 10^5 cells per ml in the incubation mixture. Aliquots of cells were added to 1.5 × 7.5 cm nitrocellulose tubes on ice. Inhibitors or other reagents were added (0.1 ml) in Krebs-Ringer buffer. Angiotensin II dissolved in Krebs-Ringer buffer was added (0.1 ml). The final incubation volume was 1 ml. Tubes were incubated at 37°C and shaken at 100 oscillations per min under 95% O_2/5% CO_2 for 2 hr.

After incubations were completed, tubes were centrifuged for 15 min at 1,000 × g, and the supernatants were removed. Aldosterone was assayed in cell supernatants by radioimmunoassay (New England Nuclear radioimmunoassay kit). Aldosterone radioimmunoassays performed directly on supernatants and on methylene chloride extracts of the supernatants gave comparable results.

RESULTS

The effect of angiotensin II (0.01 and 0.1 μM) on $^{45}Ca^{2+}$ efflux from cells that had been loaded with $^{45}Ca^{2+}$ for 25 min in Krebs buffer with no added unlabeled calcium is shown in Fig. 1. Within 5 min, 0.1 μM angiotensin II caused the efflux of 36.5% of the $^{45}Ca^{2+}$ taken up by the cells.

Fig. 2 shows the effect of angiotensin II (0.01 and 0.1 μM) on $^{45}Ca^{2+}$ influx in Krebs buffer with no added unlabeled calcium. These concentrations of angiotensin II inhibited $^{45}Ca^{2+}$ influx within 2 min. The calcium that was taken up between 0 and 5 min in the presence of 0.1 μM angiotensin II was only 24.4% of the influx under control conditions. Similar results were obtained when the total calcium concentration was 0.5 mM.

Fig. 3 shows a dose-response curve for the effects of angiotensin II on $^{45}Ca^{2+}$ influx and on steroidogenesis.

To determine whether the effects of angiotensin II in this

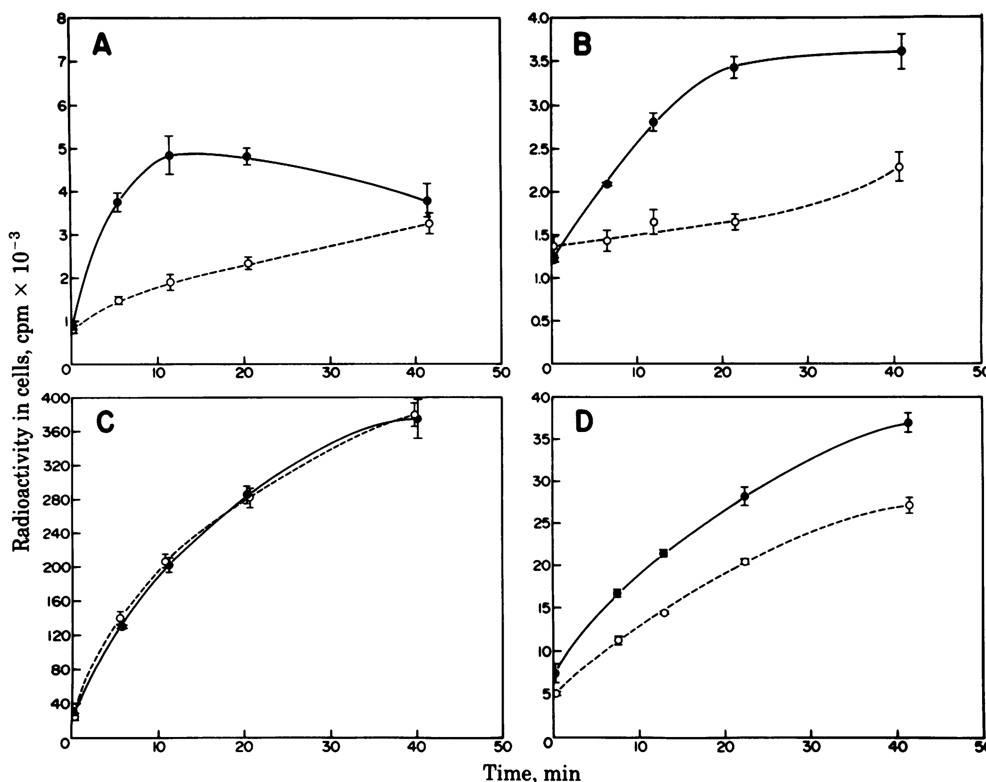

FIG. 6. Influx of four radioactive cations into glomerulosa cells in the presence (○) and absence (●) of 0.1 μM angiotensin II. Conditions were the same as for the $^{45}Ca^{2+}$ influx experiment. (*A*) $^{45}Ca^{2+}$. (*B*) $^{133}Ba^{2+}$. (*C*) $^{57}Co^{2+}$. (*D*) $^{54}Mn^{2+}$. Results with $^{51}Cr^{6+}$ were similar to those shown in *C*.

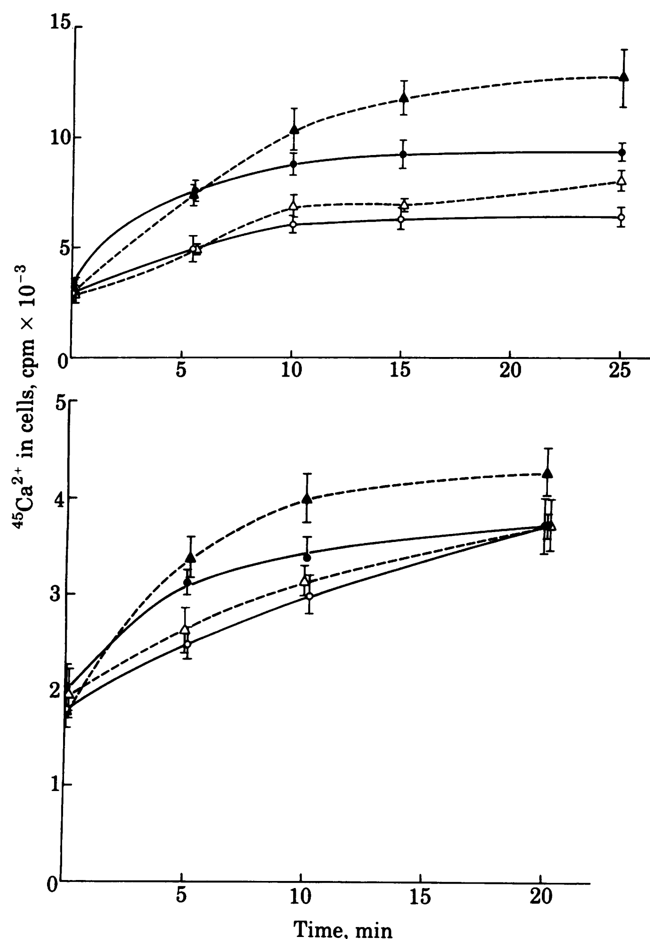

FIG. 7. (*Upper*) Effect of increased K^+ concentration on uptake of $^{45}Ca^{2+}$ by glomerulosa cells in the presence (○——○) and absence (●——●) of 0.1 M angiotensin II. ——, Incubations with the standard concentration of K^+ (3.6 mM); ---, incubations with K^+ at 23.6 mM. (*Lower*) Effect of ouabain (50 μM) on uptake of $^{45}Ca^{2+}$ by glomerulosa cells in the presence and absence of 0.1 μM angiotensin II. ○——○, Angiotensin II; ●——●, control; ▲---▲, ouabain; △---△, ouabain and angiotensin II.

system were receptor-mediated, the competitive inhibitor [1-sarcosine, 5-valine, 8-alanine]angiotensin II (Saralasin) was added. Saralasin exerted a negligible effect by itself but prevented most of the angiotensin II effect (Fig. 4). An analog of angiotensin II that is inert in most bioassay systems, des-Phe^8-angiotensin II, showed virtually no activity in this system.

Similar effects of angiotensin II were observed in either Krebs buffer with $NaHCO_3$ and NaH_2PO_4 and 95% O_2/5% CO_2 or in buffer with Hepes in place of $NaHCO_3$ and NaH_2PO_4 and an atmosphere of air. Most experiments conducted after this comparison used 10 mM Hepes instead of phosphate and bicarbonate and were run in air rather than O_2/CO_2.

When experiments were performed at 21°C, 29°C, and 37°C, $^{45}Ca^{2+}$ influx at any given time increased as a function of temperature, but angiotensin II was effective at all three temperatures. Elimination of glucose from the incubation medium in $^{45}Ca^{2+}$ influx experiments led to a slight decrease in influx, but the net effect of angiotensin II was similar with or without glucose. Elimination of magnesium increased influx, but the net effect of angiotensin II was similar with or without magnesium.

Manganese and lanthanum, which are known antagonists of calcium transport, completely inhibited $^{45}Ca^{2+}$ influx. La^{3+} also produced a dramatic efflux of preloaded $^{45}Ca^{2+}$; 2.5 mM $LaCl_3$ gave results similar to 0.10 μM angiotensin II. However, a difference persisted between control and angiotensin II-treated cells when lanthanum was added after angiotensin had exerted its effect (Fig. 5). La^{3+} acts to remove extracellular calcium, so this result suggests that at least some of the effect of angiotensin II was on intracellular calcium or the movement of calcium to intracellular pools.

When other radioactive cations were used in place of $^{45}Ca^{2+}$, influxes of $^{133}Ba^{2+}$ and $^{54}Mn^{2+}$ were affected by angiotensin II, whereas those of $^{57}Co^{2+}$ and $^{51}Cr^{6+}$ were not (Fig. 6).

One of the mechanisms by which free calcium is extruded from the cell is calcium/sodium exchange, which depends on the sodium gradient maintained by the Na^+,K^+-ATPase. Manipulations designed to affect the sodium pump did alter $^{45}Ca^{2+}$ fluxes in glomerulosa cells but did not abolish the angiotensin effect. For example, substitution of choline chloride for sodium chloride increased the control influx of $^{45}Ca^{2+}$ and accentuated the effect of angiotensin II. Increasing KCl to 23.6 mM or adding ouabain (50 μM) also accentuated the angiotensin effect (Fig. 7).

DISCUSSION

Angiotensin II produced a rapid and significant alteration in calcium fluxes in one of the hormone's primary targets, the adrenal glomerulosa. The doses of hormone required for effects on $^{45}Ca^{2+}$ fluxes and on aldosterone synthesis were similar. Results of experiments with a competitive inhibitor of angiotensin indicate that the effects of angiotensin II on calcium were receptor-mediated. Experiments with other labeled cations showed some specificity of hormone effect. Results with lanthanum suggest that the effects of angiotensin II were not limited to the calcium bound to extracellular locations. The negative results with ouabain and high K^+ concentration indicate that the angiotensin effect on calcium was not mediated by Na^+,K^+-ATPase.

Increased cytosolic calcium has been implicated in the effects of glucose on insulin release (38, 39); α-adrenergic agonists on glycogenolysis (31–35); concanavalin A on activation of lymphocytes, thymocytes (40, 41), and mast cells (42); chemotactic factor on neutrophil activation (43); and muscarinic agonists on amylase secretion from pancreatic acinar cells (44). Altered calcium fluxes have been observed in all these systems.

Because of the dependence of angiotensin action on extracellular calcium, we postulated that the hormone would increase calcium influx to increase the level of free calcium in the cytosol. The observed decrease in influx is difficult for us to rationalize.

An increase in the concentration of cystosolic calcium may be brought about by increased influx from the surrounding medium or release from intracellular stores, or both. Depending on the relative effects on plasma membrane and intracellular stores, a hormone that increases cytosolic calcium could cause either a net gain or loss of total cellular calcium. In the case of α-adrenergic stimulation of liver cells, norepinephrine increased the efflux of preloaded $^{45}Ca^{2+}$ and decreased the influx of $^{45}Ca^{2+}$ (37), similar to the results reported here. Calcium electrode measurements confirmed that there was a net loss of calcium from the cells. Work by others (35) has shown a net loss of calcium from liver mitochondria when livers were perfused with angiotensin or α-adrenergic agonists. Both observations suggested to those authors that the critical change induced by hormone was a transient rise in free cytosolic calcium, which could trigger other processes. We have no direct evidence bearing on a change that angiotensin may cause in cytosolic calcium

or on a mechanism by which changes in calcium fluxes increase steroidogenesis.

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