Insights Into the Mechanism by Which Inhibition of Na,K-ATPase Stimulates Aldosterone Production

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Inhibition of Na,K-adenosine triphosphatase (Na,K-ATPase) activity by ouabain has been shown to increase the release of aldosterone from rat glomerulosa cells, but the mechanism by which this elevation of aldosterone production occurs has not been established. Small changes in membrane potential can significantly affect aldosterone release. Consequently, inhibition of Na,K-ATPase in glomerulosa cells may stimulate aldosterone production by membrane depolarization. If so, ouabain-stimulated production should be dependent on calcium influx through voltage-gated calcium channels. It has previously been shown that ouabain induces a moderately rapid increase in cytosolic calcium in rat glomerulosa cells. Therefore, in this study, we test whether ouabain stimulates aldosterone production with a time course consistent with early membrane depolarization as suggested by the previously reported early increase in cytosolic calcium. To study the time course of aldosterone production, we developed a perfusion technique that allows an examination of the initial effects of ouabain on aldosterone production. The results show that ouabain rapidly stimulates aldosterone production. Continuous perfusion with 0.25 or 1 mmol/L ouabain induced a brisk, robust increase in aldosterone production, followed by a decrease to near baseline over 60 minutes. Ouabain-stimulated aldosterone production was dependent on the presence of extracellular calcium and calcium influx through voltage-gated calcium channels. Our results support the hypothesis that the inhibition of Na,K-ATPase in rat adrenal glomerulosa cells immediately depolarizes the membrane potential and opens voltage-gated calcium channels.

INHIBITION OF Na,K-adenosine triphosphatase (Na, K-ATPase) in rat glomerulosa cells increases aldosterone production.[1-6] The mechanism by which this occurs has not been established. Inhibition of Na,K-ATPase may stimulate aldosterone release by depolarization of the cell membrane. Small changes in membrane potential can significantly affect aldosterone production as exemplified by potassium-stimulated release. It has previously been shown that ouabain induces a moderately rapid increase in cytosolic calcium in rat glomerulosa cells that is inhibited by nifedipine.[3] This observation is consistent with the hypothesis that ouabain decreases the membrane potential, which increases cytosolic calcium by opening voltage-gated calcium channels, leading to an increase in aldosterone production. Therefore, in the experiments reported herein, we test whether ouabain stimulates aldosterone production with a time course consistent with early membrane depolarization as suggested by the previously reported early increase in cytosolic calcium. Additionally, ouabain-stimulated production should be dependent on extracellular calcium and its influx through voltage-dependent calcium channels if this hypothesis is true.

Despite the large number of studies on the effects of ouabain on aldosterone production, there are no previous studies that determined the time course by which inhibition of Na, K-ATPase affects aldosterone release. This is significant, because the effects of inhibition of Na,K-ATPase in a given cell type vary as a function of time.

In a cell where Na,K-ATPase makes a substantial direct electrogenic contribution to the resting membrane potential, pumping out three Na^+ in exchange for two K^+, inhibition of Na,K-ATPase activity will cause an immediate depolarization of the membrane potential well before any changes in the gradients of sodium and potassium. In other words, the hallmark of this effect is that it occurs suddenly as soon as Na,K-ATPase activity is terminated. Any delay in the electrogenic response would be due to the time required for ouabain to bind to most of the Na,K-ATPase. This length of time can vary considerably, depending in part on the affinity of a particular Na,K-ATPase isoform for ouabain and the concentration of ouabain. Thus, in rat adrenal cells, which contain primarily an isoform with an especially low affinity for ouabain (K_d = 60 μmol/L), one would predict that millimolar concentrations of ouabain should stimulate the release of aldosterone within a few minutes of its application, similar to the time required for ouabain to increase intracellular calcium in rat glomerulosa cells.[3] Membrane depolarization also occurs as the gradients of sodium and potassium decay, but this decline in the membrane potential is slower, with a time course of many minutes to hours, the exact rate depending on the other transport mechanisms and leak permeabilities present. In adrenal cells, a 60-minute exposure to ouabain diminishes the membrane potential by 20 to 30 mV[7,8] and intracellular potassium decreases by 50% over 2 hours.[6] As the gradients of sodium and potassium decline, the cells become progressively less able to regulate their volume, intracellular calcium, and pH.

Historically, the effects of ouabain on aldosterone production have been studied by incubating cells with ouabain for 60 to 120 minutes.[1-6] Consequently, it is difficult to determine if ouabain stimulates aldosterone production within a time frame consistent with its more rapid effect on intracellular calcium. To determine the earlier events that may occur with Na,K-ATPase inhibition, we developed a perfusion technique to examine the initial effects of ouabain on aldosterone production. The results show that ouabain induces a rapid increase in aldosterone

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production consistent with early membrane depolarization. We present additional evidence showing that ouabain-stimulated production is dependent on calcium influx through voltage-gated calcium channels. Therefore, our results support the hypothesis that the inhibition of Na,K-ATPase in rat adrenal glomerulosa cells rapidly depolarizes the membrane potential and opens voltage-dependent calcium channels.

MATERIALS AND METHODS

Adrenal Cell Preparation

Adrenal capsules including the zona glomerulosa were obtained from female Sprague-Dawley rats weighing 200 to 224 g. Glomerulosa cells were collagenase-dispersed as previously described[9] in medium 199 (GIBCO, Grand Island, NY) containing modified Earle salts (130 mmol/L NaCl, 4.0 mmol/L KCl, 10 mmol/L HEPES, Na salt, pH 7.4, 0.2% bovine serum albumin, and no bicarbonate). The cells were continuously gassed with 100% O_2. The calcium experiments were performed in medium 199 containing no added calcium.

Adrenal Experiments

Cells were either statically incubated (50,000 cells in 0.5 mL medium 199) for 60 minutes or perfused. Perfusion studies were performed with a 1-mL syringe. On the needle end was placed a filter holder (Nucleopore, Pleasanton, CA) containing a membrane filter (Filinert membrane; Costar, Cambridge, MA). In the bottom of the syringe was placed a small piece of Kimwipe (Kimberly-Clark, Roswell, GA) over which 0.1 to 0.2 mL hydrated Bio-Gel P-2 (200 to 400 mesh; Bio-Rad, Richmond, CA) was layered. The rubber end of the plunger was removed and placed 0.5 cm from the top of the Bio-Gel P-2. The rubber plunger was impaled with a needle. Medium 199 was pumped through the syringe by a peristaltic pump placed at the distal end of the tubing so that the medium was pulled through the column at a rate of 0.25 mL/min. Glomerulosa cells (500,000 to 550,000) were placed on top of the Bio-Gel. We found that layering the cells on top of the Bio-Gel produced a better aldosterone response than mixing the cells in the Bio-Gel. Samples were collected every 5 minutes, and aldosterone was quantified by radioimmunoassay[9] using an antibody to aldosterone obtained from the National Hormone and Pituitary Program, National Institutes of Health (Bethesda, MD).

Statistical Analysis

The data were analyzed by paired t test ($P < .05$, two-tailed, $df = 4$). Results are the mean ± SE.

RESULTS

Statically Incubated Cells

Initial studies were performed to determine the concentration-response to ouabain in statically incubated cells to verify that the rat glomerulosa cells in our studies produced similar responses to those previously reported. Ouabain stimulated the production of aldosterone in a concentration-dependent manner up to 100 μmol/L (Fig 1). Higher concentrations of ouabain (200 μmol/L) resulted in reduced production, and additional studies showed the response to 1 mmol/L ouabain to be 45% ± 4% ($p < .05$, n = 3 experiments) of the response to 100 μmol/L ouabain. These results are similar to those previously reported.[1-5]

Perfused Cells

Ouabain increased aldosterone production by perfused glomerulosa cells. The rapidity of the response was dependent on the perfusion concentration of ouabain. Continuous perfusion with ouabain concentrations of 250 μmol/L or greater induced an initial rapid increase in aldosterone production followed by a decline to near baseline. In contrast, perfusion with 100 μmol/L ouabain over 60 minutes led to a gradual increase in aldosterone production to a plateau during the period of observation (Fig 2).

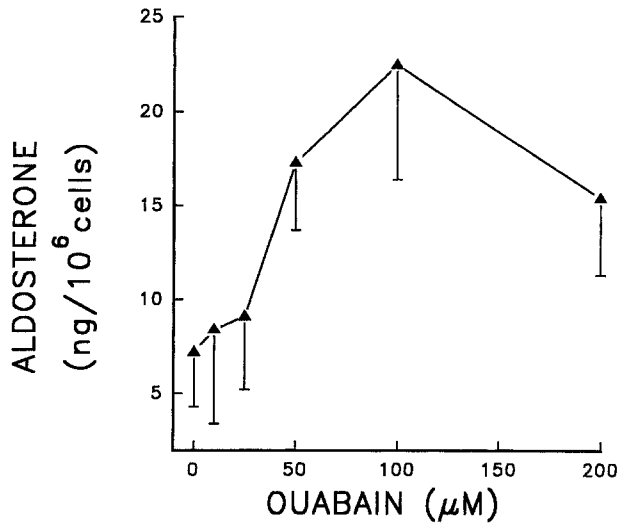

Fig 1. Effect of ouabain on aldosterone production by statically incubated rat adrenal glomerulosa cells. Aldosterone production was quantified over a 60-minute incubation with ouabain (n = 3 experiments). Results are the mean and error bars are the SE.

We compared the aldosterone response to ouabain with that of potassium, because potassium is known to depolarize the cell membrane. We used a high concentration of ouabain in these studies to inhibit Na,K-ATPase as rapidly as possible. The aldosterone response to 1 mmol/L ouabain by perfused cells

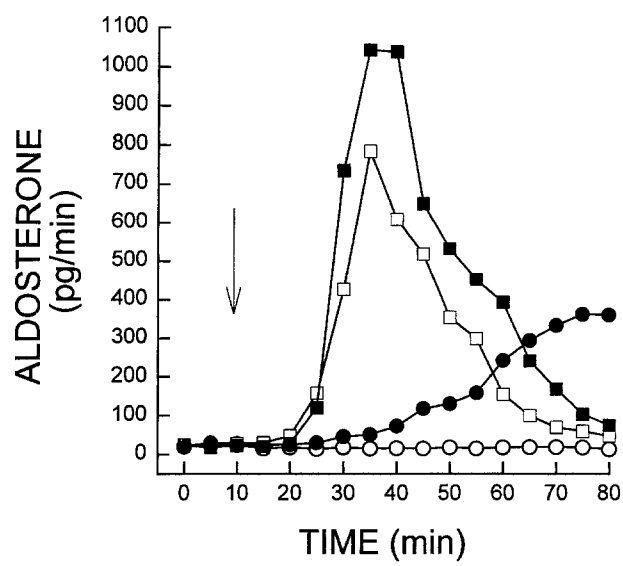

Fig 2. Effect of ouabain on aldosterone production by perfused rat glomerulosa cells. A representative study is shown from 3 independent experiments in which ouabain was continuously perfused beginning at 10 minutes (arrow). Ouabain concentrations were 0 (○), 100 μmol/L (●), 250 μmol/L (■), and 1 mmol/L (□).

was brisk, albeit slightly delayed in comparison to the response to 10 mmol/L potassium, probably due to the time required for ouabain to bind to Na,K-ATPase. Importantly, the response to ouabain declined over time, which may reflect the collapse of ion gradients (Fig 3).

Evidence That Ouabain-Stimulated Production Is Due to Membrane Depolarization

If ouabain-stimulated aldosterone production is due to membrane depolarization, the production should be dependent on the presence of extracellular calcium and be inhibitable by a calcium-channel blocker. These are requisite characteristics of other stimuli of aldosterone release that depolarize the membrane, namely potassium and angiotensin II.[10] To determine if the response to ouabain is calcium-dependent, cells were incubated with 100 μmol/L ouabain and with calcium concentrations of 0 to 4 mmol/L. With no added calcium in the buffer, ouabain failed to stimulate aldosterone production. Increasing concentrations of calcium led to greater aldosterone production in response to ouabain (Fig 4). In addition, calcium influx through voltage-gated calcium channels was required for ouabain-stimulated aldosterone production. Isradipine (a gift from Sandoz Pharmaceuticals, East Hanover, NJ), a calcium-channel blocker, diminished the production of aldosterone in response to 100 μmol/L ouabain in a concentration-dependent manner (Fig 5). Isradipine (10 μmol/L) did not block corticotropin (10 nmol/L)-stimulated aldosterone production. Basal production was 20 ± 7 ng/10^6 cells and increased to 146 ± 5 and 156 ± 33 ng/10^6 cells with and without isradipine, respectively, suggesting that inhibition was not due to nonspecific inhibition of an aldosterone biosynthetic enzyme.

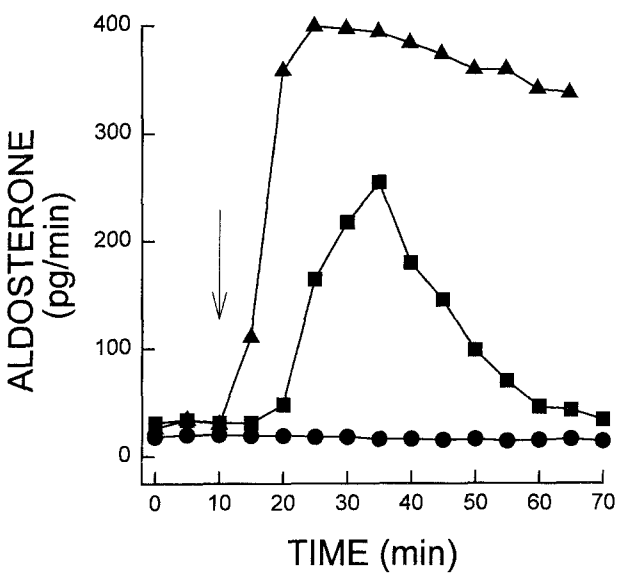

Fig 3. Comparison of aldosterone production in perfused glomerulosa cells in response to ouabain and potassium. A representative study is shown from 2 independent experiments in which cells were continuously perfused with 1 mmol/L ouabain (■), 10 mmol/L KCl (▲), or buffer (●) beginning at the arrow.

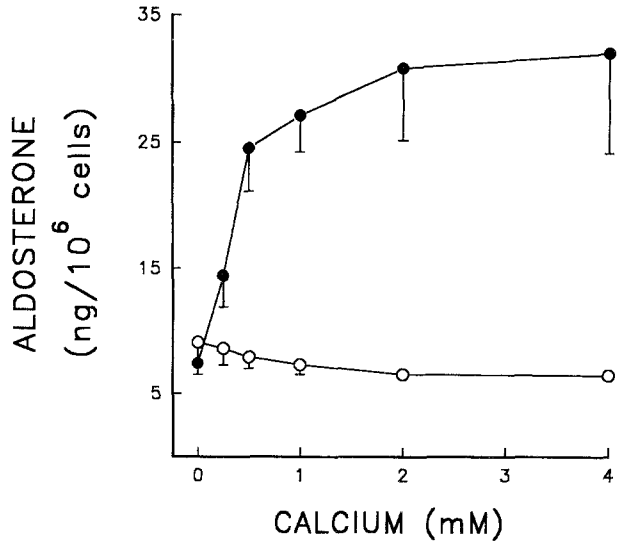

Fig 4. Effect of calcium on ouabain-stimulated aldosterone production by statically incubated glomerulosa cells. Cells were incubated with (●) and without (○) 100 μmol/L ouabain at calcium concentrations of 0 to 4 mmol/L (n = 3 experiments). Results are the mean; error bars are the SE and may be located within the symbols.

DISCUSSION

The results of this study show that ouabain stimulates aldosterone production with a time course consistent with early membrane depolarization and the previously reported early increase in intracellular calcium that is inhibited by a calcium-

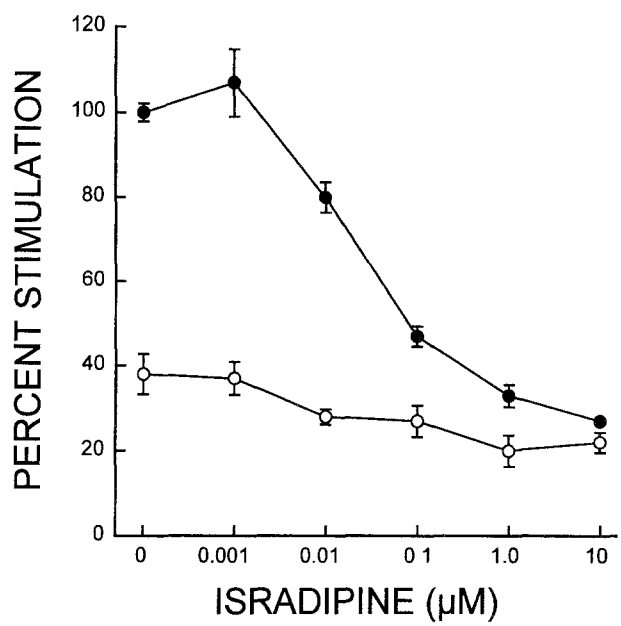

Fig 5. Effect of isradipine on ouabain-stimulated aldosterone production by statically incubated glomerulosa cells. Cells were incubated with (●) and without (○) 100 μmol/L ouabain at isradipine concentrations of 0 to 10 μmol/L. Results are expressed as a percent of aldosterone secretion in response to 100 μmol/L ouabain in the absence of isradipine (n = 3 experiments). Aldosterone production increased from 20 ± 7 to 51 ± 12 ng/10^6 cells with 100 μmol/L ouabain (mean ± SE). Error bars are the SE and may be located within the symbols.

channel blocker.[3] The evidence to support ouabain-induced membrane depolarization is the observation that ouabain stimulation is dependent on extracellular calcium and its influx through voltage-gated calcium channels. The calcium dependency of ouabain stimulation is similar to that of potassium and angiotensin II, secretagogues that depolarize the membrane.[10] Thus, the evidence collectively suggests that ouabain diminishes the membrane potential in glomerulosa cells. In addition, the evidence also suggests that the initial depolarization is due to inhibition of the electrogenic Na,K-ATPase, as reflected in the rapid rate at which high concentrations of ouabain stimulate aldosterone production. We believe it is this event which induces the initial increase in aldosterone release.

Cells perfused with 1 mmol/L ouabain responded with a brisk production of aldosterone. However, the onset of the increase in aldosterone production was delayed compared with the response to potassium (Fig 3). The delay is most likely due to the time required for ouabain to bind to the low-affinity rat α-1 isoform, the major Na,K-ATPase isoform present in rat glomerulosa cells.[3] In contrast, increasing the extracellular potassium rapidly depolarizes the membrane (measured in seconds or less), resulting in a brisk response. These events are best reflected in the rapidity with which intracellular calcium increases in response to potassium relative to ouabain. Potassium (10 mmol/L) induces a rapid increase in intracellular calcium,[11] whereas ouabain produces a much slower increase.[3] The time required for ouabain to stimulate aldosterone production in our study was approximately the same as the time required for a similar concentration of ouabain to increase intracellular calcium to stimulatory levels in the same cells under similar conditions.[3] For instance, it required 15 minutes for 250 μmol/L ouabain to stimulate aldosterone production above baseline levels (Fig 2). Over this same period, 300 μmol/L ouabain increased intracellular free calcium from approximately 110 nmol/L to 170 nmol/L.[3]

The reason for the biphasic aldosterone response to 0.25 and 1 mmol/L ouabain is unclear. The initial increase in aldosterone production is most likely due to membrane depolarization. The subsequent decline in production may be due to a loss of intracellular potassium, which has been associated with diminished aldosterone production.[6,12-14] However, the decline is unlikely due to cell death or a direct effect of ouabain on steroid biosynthesis, since the cells exclude trypan blue[12] and aldosterone production can be increased under these conditions with appropriate stimuli.[1,4]

The mechanism by which ouabain increases aldosterone production is calcium-dependent. The lack of a stimulatory effect of ouabain in the absence of extracellular calcium suggests that calcium influx is required (Fig 4). The inhibition of ouabain-stimulated aldosterone production by the calcium-channel inhibitor isradipine (Fig 5) suggests that calcium enters through voltage-gated calcium channels. In addition, these latter studies suggest that calcium influx via the Na^+/Ca^{2+} exchanger plays little or no role in ouabain-stimulated aldosterone production. These findings are consistent with the observation that ouabain depolarizes the cell membrane of glomerulosa cells.[8] They are also consistent with the previous observation that ouabain increases cytosolic calcium in glomerulosa cells via voltage-dependent calcium channels.[3]

Our findings are also in agreement with those of Schiffrin et al,[2] who found that lanthanum, a trivalent calcium antagonist, and verapamil block ouabain-stimulated aldosterone production. However, we found that 100 μmol/L verapamil, the concentration used in their studies, directly blocks aldosterone synthase.[15] In addition, lanthanum blocks Na,K-ATPase activity[16,17] and thereby, in combination with the low stimulatory dose of ouabain, could behave like high-dosage ouabain, resulting in no increase in aldosterone release.[2] For these reasons, we chose to study the effect of ouabain in the absence of extracellular calcium using a different class of calcium-channel blocker that does not block enzymes in the aldosterone biosynthetic pathway.

Other investigators have examined the effect of ouabain on aldosterone production by glomerulosa cells from a variety of species, with mixed results.[1-6,18-23] The conflicting results may be due to the complex nature of Na,K-ATPase inhibition with ouabain, which is dependent on the animal species and the ouabain concentration and duration of exposure. The ouabain binding affinity for Na,K-ATPase varies widely between species and between isoforms of Na,K-ATPase. The rat α-1 isoform, the predominant form in rat glomerulosa cells,[3] has a low affinity for ouabain (K_d ~60 μmol/L), whereas this same isoform in the dog has an affinity that is several orders of magnitude higher.[24] In addition, the effect of ouabain on Na,K-ATPase activity is dependent on the ouabain concentration used relative to the ouabain binding affinity for Na,K-ATPase in the species under study. Finally, the effect of ouabain on Na,K-ATPase activity is dependent on the length of exposure to ouabain. Longer periods increase the amount of Na,K-ATPase inhibition and compound the cellular changes associated with membrane depolarization and altered ion gradients.

In conclusion, the initial increase in aldosterone production in response to ouabain is stimulatory in the rat and is most likely due to membrane depolarization caused by inhibition of the electrogenic contribution of Na,K-ATPase to the membrane potential. Inhibition of Na,K-ATPase over an extended period leads to a diminished aldosterone response, which may reflect a collapse in the ion gradients. The results of this study suggest that inhibition of Na,K-ATPase by the recently described ouabain-like factor originating from glomerulosa cells[25-27] has the potential to stimulate aldosterone production or modify the responsiveness of glomerulosa cells to the stimuli of aldosterone release.

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