The Role of Calcium in the Control of Renin Release
ALLEN J. NAFTILAN, M.D., PH.D., AND SUZANNE OPARIL, M.D.

SUMMARY The effects of removing external calcium and inhibiting entry of calcium into the cell by treatment with D-600 on renin release from renal cortical slices of male Sprague-Dawley rats were examined. Baseline renin release, angiotensin II (All)-induced inhibition, and isoproterenol-induced stimulation of renin release were studied. Removal of external calcium by chelation with 5 mM EGTA inhibited basal renin release while treatment with 1 mM EGTA stimulated basal renin release slightly. Incubation of slices with zero calcium medium containing 1 mM EGTA had no effect on isoproterenol-induced stimulation of renin release. In contrast, similar treatment reduced the inhibitory effect of All from 58.7% of baseline to 85.3% (p < 0.001). Similarly, blockage of calcium entry into cells with D-600 had no effect on isoproterenol-induced stimulation of renin release but abolished All-induced inhibition. Replacement of sodium in the bathing medium with choline had no effect on baseline renin release or on All-induced inhibition of renin release, ruling out the possibility that D-600 altered renin release via an effect on sodium influx. Taken together, the data strongly suggest that All-induced inhibition of renin release is partially dependent on the presence of external calcium but that isoproterenol-induced stimulation of renin release is not.

KEY WORDS • renin release • angiotensin II • calcium • isoproterenol • D-600

THE role of calcium in the control of renin release is incompletely understood. In vivo experiments have yielded conflicting results in that calcium infusion has been shown to inhibit renin release in some studies1^2 and stimulate renin release in others.3 Using the isolated perfused rat kidney preparation, Peart and colleagues4 5 found that removal of external calcium from the perfusate by chelation with EDTA stimulated renin release and blocked the inhibitory effect of angiotensin II (All) on renin release but did not alter isoproterenol-induced stimulation of renin release. They further observed that the calcium ionophore A23187 inhibited renin release.6 In a similar perfused rat kidney preparation, Fray and Park7 demonstrated that the renin-stimulating effects of a variety of agents were attenuated by raising extracellular calcium levels and that the effects of inhibitory stimuli were enhanced by raising extracellular calcium.8 From these results Fray9 has generalized that agents that inhibit renin release increase intracellular calcium levels while stimulators of renin release lower calcium concentrations.

Using the rat renal cortical slice preparation, we10 11 have shown that All inhibits renin release from renal cortical slices in a dose-dependent manner via a direct action on the juxtaglomerular cell and that isoproterenol stimulates renin release. In the current study we have extended these observations by exploring the role of calcium in mediating renin release from rat renal cortical slices. We examined the effects of removing external calcium and inhibiting entry of calcium into the cell by treatment with D-600 on baseline renin release and on All-induced inhibition and isoproterenol-induced stimulation of renin release. From these results and those reported previously by others, we have constructed a model for the role of calcium in the intracellular mechanism of renin release.

Materials and Methods
Kidney Slice Preparation
Renal cortical slices were prepared as described earlier.10 11 For experiments in which the effects of All were examined, human serum albumin was added to the medium (1 mg/ml, final concentration) to prevent the peptides from adhering to glass. All was synthesized in our laboratory as described previously.12 All was dissolved in 0.05 N acetic acid at a concentration of 5 mM. When isoproterenol was added to the slices, ascorbic acid (6 × 10−3 M, final concentration) was included to prevent oxidation. The medium was then readjusted to pH 7.4 with 5 N NaOH. Isoproterenol (Sigma) was dissolved in a minimal volume of medium.

Each slice was incubated in 2 ml of medium for 15 minutes at 37°C under 95% O2-5% CO2 in a shaking water bath, the medium was discarded and the slice washed with an additional 3 ml of medium. After the initial wash, slices were incubated for five consecutive 15-minute periods (A, B, C, D, and E) in 2 ml of medium. At the end of each period the medium was aspirated and placed in a chilled tube to which 20 μl of 8-hydroxyquinoline (0.34 M) and 100 μl of EDTA

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(4% wt/vol) were immediately added and the tube was gently mixed. At the end of Period E, each slice was frozen in a dry ice/acetone bath, lyophilized, and weighed.

Baseline renin activity was taken as the mean of Periods A and B. All or isoproterenol was added prior to Period C and aspirated at the end of the period so that the slice was exposed to either agent for only 15 minutes. Renin activity measured during Periods C, D, and E was expressed as a percent of the control level and taken as an index of the All or isoproterenol effect. Renin activity during Periods C, D and E was compared to baseline renin activity by the Student's paired t test.

Renin Release Experiments

To explore the role of extracellular calcium in the control of renin release, two sets of experiments were conducted. In the first study, slices were incubated in Krebs bicarbonate buffer containing 1.27 mM calcium during Periods A and B. Prior to Period C, the slices were washed once with 2 ml of "calcium-free" medium containing zero calcium with EGTA added (1 or 5 mM, final concentration) to chelate trace calcium. The slices were then incubated in 2 ml of this medium during Period C. In Periods D and E, the slices were incubated in standard Krebs bicarbonate medium (1.27 mM calcium). The effects of this treatment on basal renin release and on All-induced inhibition and isoproterenol-induced stimulation of renin release were examined.

In a second study, the effects of the calcium antagonist D-600 on renin release were evaluated. D-600 was dissolved in absolute ethanol (20 μg/ml, final concentration) and 20 μl was added to the medium for the initial wash and for all five experimental periods. In these experiments All or isoproterenol was added prior to Period D rather than Period C, and baseline renin release was taken as the mean of Periods A, B, and C. A 60-minute preincubation with D-600 was necessary because of its slow onset of action.14,15 Pilot studies had shown that if a shorter period of incubation with D-600 were used, renin release was unaffected. The effects of D-600 on All-induced inhibition and isoproterenol-induced stimulation of renin release were examined as described above.

Since D-600 can block both calcium and sodium channels,14,15 the effects of lowering external sodium concentration from 130 to 10 mM by replacing the sodium chloride in the Krebs bicarbonate medium with choline chloride were examined in a third study. Baseline renin release and All-induced inhibition of renin release were compared in normal (130 mM) and low (10 mM) sodium media.

Statistical Methods

Standard statistical methods, including paired and unpaired Student's two-tailed t tests, were used where indicated in the analysis of data.16

Results

Removal of External Calcium

The effect on basal renin release of incubating renal cortical slices in zero calcium medium containing 5 mM EGTA is shown in figure 1. Removal of external calcium and exposure to 5 mM EGTA resulted in a small but significant reduction in basal renin release in both Periods C and D when compared to baseline release rates (Periods A and B) by the paired t test. To minimize changes in membrane permeability caused by chelation of divalent cations, the final concentration of EGTA was lowered to 1 mM. This concentration of chelator was then used in all further experiments. This treatment resulted in a slight stimulation of basal renin release (to 125% of baseline) during Period C only (p < 0.05) (table 1).

The effects of incubating renal cortical slices with zero calcium medium containing 1 mM EGTA on both isoproterenol-induced stimulation and All-induced inhibition of renin release were then investigated. As seen in figure 2, removal of external calcium and exposure to 1 mM EGTA had no effect on isoproterenol-induced stimulation of renin release. The 10−5 M dose of isoproterenol was selected because dose-response studies showed that this dose produced consistent but submaximal stimulation of renin release in the renal cortical slice preparation. During Period D, the period of maximal stimulation, renin release was increased to 170% of control in 1.27 mM calcium and 180% of control in zero calcium + 1 mM EGTA. In contrast, removal of external calcium and exposure of the slices to 1 mM EGTA resulted in a blunting of the inhibitory effect of All on renin release (table 1 and fig. 3). The 5 × 10−5 M dose of All was chosen because dose-response studies showed that this dose produced consistent but submaximal inhibition of renin release.

![Figure 1](https://example.com/fig1.png)

**Figure 1. Effects of zero calcium medium containing 5 mM EGTA on baseline renin release. Results are expressed as a percentage of control ± SEM. The P values represent comparisons with the mean of Periods A and B using Student’s paired t test. N.S. = not significant.**
During Period D, the period of maximal inhibition, all lowered renin release to 58.7% of baseline levels in the presence of 1.27 mM calcium and to 85.3% of baseline levels in zero calcium + 1 mM EGTA (p < 0.001).

Effects of Calcium Antagonists
To examine the effects of D-600 on renin release, the agent had to be present throughout all five incubation periods because of its slow onset of action. Baseline renin release was stable from Period A to E with 1.27mM Ca++ (n=20) and without Ca++ + 1mM EGTA (n=8).

D-600 (20 ng/ml, final concentration) present in the medium, indicating that D-600 alone had no effect on baseline renin release. Consistent with the results of experiments performed with zero calcium, the presence of D-600 had no effect on isoproterenol-induced stimulation of renin release (fig. 4, Panel B). Renin release was stimulated to a level 135% of baseline without D-600 and 147% of baseline with D-600 present. Again, the 10^-5M dose of isoproterenol was chosen for the reasons cited above. In contrast, D-600 did not affect renin release.
CALCITONIN AND RENIN RELEASE

Discussion

These experiments have shown that removal of external calcium by chelation with EGTA had varying effects on baseline renin release depending on the concentration of EGTA used. Exposure to 5 mM EGTA resulted in a reduction in basal renin release, while 1 mM EGTA stimulated basal renin release slightly. To minimize possible changes in membrane permeability due to chelation of divalent cations, the concentration of EGTA used in studies of the effects of All and isoproterenol on renin release was reduced to 1 mM. Removal of external calcium by chelation with 1 mM EGTA attenuated the inhibitory effect of All on renin release without altering isoproterenol-induced stimulation of renin release. These findings were substantiated by the results of experiments with D-600, which clearly showed that treatment of kidney slices with D-600 almost completely abolished All-induced inhibition of renin release, but did not alter isoproterenol-induced stimulation. Taken together, the data strongly suggest that All-induced inhibition of renin release is partially dependent on the presence of external calcium but that isoproterenol-induced stimulation of renin release is not.

Freer and colleagues have shown that in some smooth muscle preparations and in cardiac muscle the action of All is dependent on an influx of external calcium. Since the juxtaglomerular cells appear to be modified smooth muscle cells, a common calcium dependent mechanism for the action of All is likely. An increase in intracellular calcium caused by an influx of external calcium may mediate both All-induced inhibition of renin release and All-induced stimulation of smooth muscle contraction.

In interpreting the experiments in which chelating agents were used to remove external calcium, two possible nonspecific effects of the chelators must be taken into account. Chelation of divalent cations could inhibit binding of All to the juxtaglomerular cell and thus attenuate or abolish All-induced inhibition of renin release. Data on the effects of chelating agents on All

<table>
<thead>
<tr>
<th>Period A</th>
<th>Period B</th>
<th>Period C</th>
<th>Period D</th>
<th>Period E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>125.3 ± 11.2</td>
<td>102.6 ± 10.2</td>
<td>128.9 ± 14.6</td>
<td>102.6 ± 10.2</td>
</tr>
<tr>
<td>Zero calcium + 1 mM EGTA (n = 12)</td>
<td>(202 ± 36.1)</td>
<td>(238 ± 35.6)</td>
<td>(203 ± 35.4)</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II (5 × 10⁻⁵ M) 1.27 mM calcium (n = 18)</td>
<td>81.2 ± 3.1</td>
<td>58.7 ± 3.0</td>
<td>66.1 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II (5 × 10⁻⁵ M) Zero calcium + 1 mM EGTA (n = 11)</td>
<td>97.4 ± 5.4</td>
<td>85.3 ± 5.8</td>
<td>70.8 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Unpaired t test comparing angiotensin II with and without calcium</td>
<td>NS</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as a percentage of baseline levels ± SEM. Numbers in parentheses are absolute values expressed as ng angiotensin I generated/hr/mg dry weight.

*p values refer to paired t tests comparing renin release during Periods C, D, and E to baseline levels. NS = not significant.

**Effects of Lowering External Sodium Concentration**

As shown in figure 5, lowering external sodium concentration from 130 to 10 mM by replacing the sodium chloride in the medium with choline chloride had no effect on either baseline renin release or All-induced inhibition of renin release during Period D. There was a small but significant attenuation of All-induced inhibition of renin release during Period E. Measurement of sodium concentration of the aspirated medium from all five periods confirmed that the sodium concentration did not fluctuate during the course of the experiment.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** Effects of reduction of sodium concentration from 130 to 10 mM by replacement of sodium chloride with choline chloride on angiotensin II-induced (5 × 10⁻⁵ M angiotensin II) inhibition of renin release. Results are expressed as a percentage of control ± SEM. The p values below the bars represent comparisons with means of Periods A and B for each group using Student's paired t test; p values above bars represent comparisons between 10 and 130 mM sodium within a given period. N.S. = not significant.
binding to receptors are conflicting. Glossman et al.\textsuperscript{20} reported that a zero calcium medium containing 5 mm EDTA had no effect on the binding of All to an adrenal cortical receptor preparation. In contrast, Bianc et al.\textsuperscript{21} reported that removal of calcium and the addition of 5 mm EDTA almost completely abolished while the addition of 5 mm EGTA severely attenuated All binding to rat renal glomeruli. Differences in receptor binding assays and differing tissue preparations could well account for these apparent conflicts in receptor binding data. Since it is not possible to measure All binding to juxtaglomerular cells in renal cortical slices, it was not possible to assess the effects of chelating agents on All binding in our experiments.

The membrane effects of chelating agents may alter renin release in a nonspecific fashion. EGTA in the range of 0.5 to 5 mM chelates membrane calcium and causes membrane depolarization.\textsuperscript{22, 23} Katholi et al.\textsuperscript{24} demonstrated that canine false tendon cells incubated with 2.0 mM EGTA depolarized within 30 minutes. A 3 mM concentration of EDTA, which is less selective than EGTA in its affinity for calcium and binds magnesium as well, caused depolarization within 5 minutes. After 30 minutes of exposure to EDTA, the cells were permanently depolarized and lanthanum was able to cross the cell membrane. This may be related to EDTA-induced separation of certain cardiac intracellular junctions.\textsuperscript{25}

The membrane effects of the chelating agents may help to explain the differences between the effects of 1 and 5 mM EGTA on baseline renin release observed in our previous experiments. Treatment of slices during Period C with 1 mM EGTA was associated with stimulation of renin release during Period D and a return to control levels during Periods D and E. Similar treatment with 5 mM EGTA inhibited renin release during Periods C and D and delayed the return to control levels until Period E. The higher concentration of EGTA may have caused greater membrane damage, resulting in inhibition of the active release of calcium from the cell and a diminution in renin release. Use of a 3 mM concentration was raised. From these studies Fray concluded that agents that inhibit renin release act by decreasing calcium permeability and elevating intracellular calcium levels and stimulation of renin release.

Despite the use of low concentrations of chelator (1 mM) and a short incubation time (15 minutes), it was not possible to rule out the possibility that the EGTA altered renin release by a membrane effect or by inhibiting binding of All to the juxtaglomerular cell. For this reason, experiments were carried out using the calcium antagonist D-600. The studies using D-600 clearly demonstrated that blockade of calcium entry into the cell almost completely abolished the inhibitory action of All without an effect on isoproterenol-induced stimulation of renin release. These studies were done using a racemic mixture of D-600 since the optical isomers were not available. As reported by Bayer et al.,\textsuperscript{14, 15} the (+) optical isomer of D-600 has a specific effect on the fast inward sodium current while the (−) optical isomer is a specific blocking agent for the slow calcium channel. It is thus possible that the D-600 used in these experiments could have altered renin release by an action on sodium influx. The observation that lowering the concentration of sodium in the medium by a factor of 10 did not alter All-induced inhibition of renin release during period D makes this unlikely. The small reduction in All-induced inhibition of renin release was seen only during Period E. As noted here and in our earlier studies,\textsuperscript{10, 11} the period of maximal effect of All or isoproterenol is during Period D. Values for Period D were used in all of our comparisons.

The finding that beta adrenergic stimulation of renin release with isoproterenol is not dependent on extracellular calcium confirms the observations of Vandongen and Peart\textsuperscript{4} in the isolated perfused rat kidney and of Lester and Rubin\textsuperscript{26} in the isolated cat kidney. In contrast, Park and Malvin\textsuperscript{27} found that incubation of pig renal cortical slices with 5 mM EDTA or 5 mM EGTA for 60 minutes blocked epinephrine-induced stimulation of renin release. The refractoriness to epinephrine observed by these investigators could be accounted for by membrane damage produced by prolonged incubation of cortical slices with relatively high concentrations of the chelating agents. Thus, the bulk of evidence suggests that an influx of external calcium is not necessary for beta adrenergic stimulation of renin release.

The data reported here are consistent with the findings of Fray and coworkers\textsuperscript{23–25} in the isolated perfused rat kidney preparation. They have shown that the renin-inhibiting effect of increased renal perfusion pressure was enhanced by raising the extracellular fluid calcium concentration and blocked by verapamil. High concentrations of extracellular potassium, which depolarized the juxtaglomerular cell,\textsuperscript{28} inhibited renin secretion and completely abolished the stimulatory effects of renal hypertension and renal vasoconstriction on renin secretion. These effects were blocked by verapamil or removal of external calcium. Further, when calcium was removed from the perfusion medium, renin secretion was stimulated. Reintroduction of calcium restored renin release nearly to normal levels.

Finally, lowering renal perfusion pressure stimulated renin release only when calcium was present in the extracellular fluid and became less effective as calcium concentration was raised. From these studies Fray concluded that agents that inhibit renin release act by depolarizing the juxtaglomerular cell, increasing calcium permeability and elevating intracellular calcium levels, and that agents that stimulate renin release do so by hyperpolarizing the juxtaglomerular cell membrane, decreasing calcium permeability, and lowering intracellular calcium levels.

Further support for this conclusion has been reported by Churchill\textsuperscript{29} using the rat renal cortical slice preparation. He found that high concentrations of extracellular potassium (60 mM) inhibited renin release but that this effect could be abolished by the addition of D-600 to the 60 mM potassium medium. He also found that the inhibitory effect of All on renin release could...
be abolished by removing the external calcium and adding 2 mM EGTA. In contrast to the results reported here, Churchill found that D-600 in concentrations of 1–3 × 10^{-5} M did not block the inhibitory effects of All on renin release. These doses were sixfold higher than those needed to block the action of high extracellular potassium. From this Churchill suggests that All must activate a calcium channel that is independent of the voltage-sensitive calcium channel stimulated by depolarizing potassium. Churchill’s experiments differed from ours in that he did not preincubate his slices with D-600. We have found, as have others, 14, 15 that D-600 has a slow onset of action. Without a 60-minute preincubation, D-600 also failed to block All-induced inhibition of renin release in our experiments. It is entirely plausible, as Churchill has suggested, that there are two sets of calcium channels with differing sensitivities to calcium channel blockers.

These data are compatible with the model of the role of calcium in the molecular mechanism of renin release suggested by Fray. 9 Inhibitors of renin release such as All or high perfusion pressure act by depolarizing the juxtaglomerular cell membrane, resulting in an influx of extracellular calcium and an increase in intracellular calcium levels. These effectors are therefore dependent on the presence of extracellular calcium and can be blocked by D-600 or verapamil. Stimulators of renin release such as beta adrenergic stimulation or low perfusion pressure hyperpolarize the juxtaglomerular cell membrane, resulting in a lowering of intracellular calcium. These effectors are not dependent on the presence of extracellular calcium. If intracellular calcium levels are lowered by removal of external calcium and an efflux of calcium from the cytoplasm, baseline renin release is stimulated. It is interesting to note that this role of calcium may be exactly opposite to that first described by Douglas 13 for release of catecholamines from adrenal chromaffin cells and subsequently described for numerous other secretory systems. 17

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