Inhibition of Aldosterone Production by Pinacidil In Vitro

J. Howard Pratt, James K. Rothrock, and Jesus H. Dominguez

Pinacidil, an antihypertensive agent that opens potassium channels, lowers plasma aldosterone levels in hypertensive patients by an unknown mechanism. In the present study, pinacidil's direct effects on production of aldosterone were assessed using isolated cells from bovine adrenal glomerulosa. Pinacidil was found to inhibit aldosterone production, both basally and during stimulation with either potassium, angiotensin II (Ang II), or adrenocorticotropic hormone (ACTH), with half maximal inhibition occurring at \(10^{-5}\) M. As assessed by the exclusion of trypan blue from cells, pinacidil did not inhibit secretion through injurious effects on glomerulosa cells. Also, washing of cells previously exposed to pinacidil restored secretory responsiveness. Pinacidil did not alter cytosolic calcium (Ca\(^{2+}\)) concentrations when aequorin was used as a photoluminescent indicator of Ca\(^{2+}\) levels, suggesting that pinacidil acted by a non-Ca\(^{2+}\)-mediated mechanism. Consistent with direct inhibition of the late pathway in steroidogenesis was that pinacidil decreased conversion of pregnenolone and corticosterone to aldosterone. Pinacidil did not block binding of Ang II to its receptor, nor did it appear to affect adrenocorticotropic hormone-receptor binding, since stimulation by cyclic AMP, the post-receptor second messenger of adrenocorticotropic hormone, was also inhibited. In summary, pinacidil inhibited directly the adrenal's production of aldosterone. The mechanism whereby the inhibition occurred was unclear. (Hypertension 1991;18:529–534)
saline. A Stadie-Riggs microtome was used to remove the outer 0.5 mm of cortex that contained the zona glomerulosa. The inner 0.5 mm of subcapsular cortex was used to harvest zona fasciculata cells. Cortical tissues were digested in 2 mg/ml collagenase and 25 μg/ml type II DNase at 37°C for 1 hour. Isolated cells were washed three times in Krebs' bicarbonate buffer.

The effect of pinacidil on aldosterone production was examined under basal conditions and during stimulation with either K⁺ (12 mM), 10⁻⁷ M Ang II, 10⁻⁷ M ACTH, or 10⁻⁵ M 8-bromo-cAMP. Pinacidil, 2.63 mg (molecular weight 263), was dissolved in 200 μl methanol and then taken up in 10 ml Krebs' bicarbonate buffer. The final concentration of methanol was adjusted to 0.2% (vol/vol) for all incubation media, although this concentration of methanol had no effect on aldosterone secretion. The final concentrations of pinacidil examined were 10⁻⁷ to 10⁻⁴ M.

Cells were preincubated for 30 minutes at 5.0 mM K⁺ and then transferred to fresh medium where they were incubated with pinacidil for 15 minutes before the addition of the stimulus. The final incubation, with or without the addition of the stimulus, was carried out for 60 minutes at 37°C in a 95% O₂-5% CO₂ mixture. Similar experiments were performed after the addition of either pregnenolone (500 ng/ml) or corticosterone (250 ng/ml) as precursors to aldosterone. The incubation medium also contained 10⁻⁴ M cycloheximide, and no stimulus was added. Fasciculata cells were used to assess the effects of 10⁻⁶ and 10⁻⁵ M pinacidil on cortisol production.

The reversibility of the effect of pinacidil on aldosterone secretion was examined by incubating glomerulosa cells with 10⁻⁵ M pinacidil for 60 minutes and then removing the pinacidil by centrifugation and exposing the glomerulosa cells to 10⁻⁷ M Ang II.

Aldosterone was quantified by radioimmunoassay using antisera fromDiagnostic Products Corp., Los Angeles, Calif., and cortisol was assayed using the Diagnostic Products Coat-a-Count radioimmunoassay kit.

**Angiotensin II Binding Experiments**

The effect of pinacidil on Ang II binding to glomerulosa cells was studied in Ca²⁺-free Krebs' bicarbonate buffer with 0.1 mM EGTA to minimize Ang II degradation. The effect of 10⁻⁶ to 10⁻⁴ M pinacidil on displacement of labeled Ang II by 10⁻⁷ to 10⁻⁵ M Ang II was tested. Incubations were carried out for 10 minutes, and then cells were centrifuged. The displacement of labeled Ang II bound to glomerulosa cells was used as an indicator of Ang II binding.

**Aequorin Experiments to Assess Changes in Cytosolic Ca²⁺**

Bovine adrenal cells were prepared and then loaded with the Ca²⁺-stimulated luminescent probe, aequorin. Cells were suspended in agarose gel and then perfused at a rate of 1.0 ml/min at 37°C. The amount of aequorin light emitted at the end of the experiment after lysis of cells with 5% Triton X-100 solution was recorded as maximal luminescence (Lmax). The concentration of cytosolic Ca²⁺ was estimated from the ratio of light observed during the course of the experiment (L0) and Lmax (L0/Lmax, or fractional luminescence). The log of L0/Lmax has been shown to be proportional to the level of Ca²⁺.

Pinacidil in concentration of 10⁻⁷ to 10⁻³ M was added to the perfusate to determine its effects on cytosolic Ca²⁺.

**Statistical Analysis**

The effects of a range of pinacidil concentrations on aldosterone secretion were examined by analysis of variance. Where appropriate the nonpaired t test was used. Results are presented as the mean±SEM.

**Results**

Pinacidil caused a dose-dependent inhibition of aldosterone secretion under basal conditions (p<0.001) (Figure 1) and during Ang II-, K⁺-, or ACTH-stimulated aldosterone secretion (p<0.001 for each stimulus) (Figure 2). Regardless of the stimulus, secretion rates were reduced by more than half with 10⁻⁵ M pinacidil. Pinacidil at concentration of 10⁻⁶ and 10⁻⁵ M did not affect basal production of cortisol but did inhibit 10⁻⁷ M ACTH-stimulated production. In the presence of ACTH but no pinacidil, cortisol production was 159±3.5 (SEM) ng/min/10⁶ cells; after the addition of 10⁻⁴ and 10⁻³ M pinacidil, cortisol production was 144.0±2.8 (p=0.000 when compared with ACTH alone) and 125.6±6.13 ng/min/10⁶ cells (p=0.004), respectively.

In Table 1 are shown effects of pinacidil on aldosterone production after the addition of precursor steroids. Conversion of pregnenolone to aldosterone was inhibited by 10⁻⁵ M pinacidil, as was conversion of corticosterone to aldosterone, indicating that inhibition by pinacidil occurred at a late step in aldosterone biosynthesis.

No toxic effect of pinacidil was observed as assessed by trypan blue exclusion (more than 90% of glomerulosa cells excluded trypan blue). The effect of a 60-minute exposure to pinacidil on the subsequent stimulatory response to Ang II is shown in Figure 3.
All, angiotensin II; K, potassium ion; ACTH, adrenocorticotropic hormone.

A 60-minute exposure of adrenal cells to 10^{-5} M pinacidil decreased basal secretion of aldosterone (p<0.001). Then, after replacing the medium containing pinacidil with medium containing 10^{-7} M Ang II, there was a fourfold increase in aldosterone secretion (p<0.001). Thus, inhibition of secretion by pinacidil was reversible and did not result from a toxic effect of pinacidil on cells. The secretory response to 10^{-6} M Ang II was in fact significantly greater when cells had been first incubated with pinacidil (p<0.001).

In Ang II binding studies, pinacidil concentrations as high as 10^{-5} M were without effects on binding of Ang II to its receptor (Figure 4). Pinacidil also inhibited 8-bromo-cAMP-stimulated secretion (Figure 5), and thus ACTH-mediated stimulation, which uses CAMP as a second messenger, was inhibited beyond the point of interaction with the ACTH receptor.

Cytosolic Ca^{2+} levels depicted as the log L_0/L_{max} of the luminescence generated by glomerulosa cells preloaded with aequorin are shown in Figure 6. Increasing extracellular K^{+} from 5 to 12 mM raised the level of cytosolic Ca^{2+}. The addition of nitrendipine (10^{-5} M) led to inhibition of Ca^{2+} influx through VSCCs (Figure 6, top panel), whereas 10^{-5} M pinacidil had no such effect on cytosolic Ca^{2+}. In addition, 10^{-5} M pinacidil produced no noticeable effect on the typical Ang II-induced Ca^{2+} transient (results not shown).

Discussion

In the present study, pinacidil inhibited aldosterone production in vitro under basal conditions as well as during stimulation with either Ang II, K^{+}, or ACTH. Such an effect on the adrenal could explain the lower plasma aldosterone levels in patients receiving pinacidil as treatment for hypertension. How pinacidil inhibited aldosterone production was, however, unclear.

Both K^{+} and Ang II stimulate aldosterone secretion by increasing cytosolic Ca^{2+}. K^{+} stimulates secretion by depolarizing glomerulosa cells, thereby increasing Ca^{2+} influx through VSCCs, Ang II, through the hydrolysis of phospholipids, releases

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**TABLE 1. Effect of Pinacidil on Aldosterone Production (pg/10^{6} cells) in the Presence of the Precursors Pregnenolone and Corticosterone**

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Control</th>
<th>10^{-5} M Pinacidil</th>
<th>10^{-5} M Pinacidil</th>
<th>Absences of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
<td>171±10</td>
<td>. . .</td>
<td>109±5</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1,567±88</td>
<td>1,186±50†</td>
<td>616±32*</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM.

*p=0.000 when examined in relation to control values.
†p=0.002 when examined in relation to control values.
cytosolic sequestered Ca\textsuperscript{2+}, but like K\textsuperscript{+}, Ang II also increases influx of Ca\textsuperscript{2+} through VSCCs.\textsuperscript{13-15} Thus, both K\textsuperscript{+} and Ang II would be expected to have their stimulatory potentials affected adversely by a K\textsuperscript{+}-channel opener such as pinacidil. However, from a series of experiments that were carried out, it was not possible to demonstrate such an effect of pinacidil on adrenal glomerulosa secretory function.

First, using the photoluminescent probe aequorin, we could show no effect of pinacidil on cytosolic Ca\textsuperscript{2+} with either K\textsuperscript{+} or Ang II as the stimulus. Considering the strong inhibition of aldosterone secretion that occurred with this concentration of pinacidil, we would have anticipated an observable decline in cytosolic Ca\textsuperscript{2+}. Second, pinacidil inhibited the conversion of pregnenolone as well as corticosterone to aldosterone, inhibition at a late step in biosynthesis, consistent with inhibition of 18-hydroxylation. The late synthetic pathway lies within mitochondria,\textsuperscript{16} which are presumably isolated from signaling events at the outer cell membrane. Such observations indicate a more direct inhibition of enzymatic activity as opposed to an effect of pinacidil to hyperpolarize the cell membrane. Third, stimulation by ACTH is coupled principally to cAMP,\textsuperscript{10} and not to increases in cytosolic Ca\textsuperscript{2+}. That pinacidil also suppressed stimulation by ACTH further suggested that pinacidil did not act by reducing voltage sensitive Ca\textsuperscript{2+} influx.

Although we could not substantiate an action for pinacidil that was consistent with its reported ability to open K\textsuperscript{+} channels, this does not refute completely the possibility that pinacidil inhibited by affecting the efflux of K\textsuperscript{+}. Effects on cytosolic Ca\textsuperscript{2+}, for example, may have
been too small or too restricted to critical areas near the cell membrane for us to observe changes with the techniques used. Also, whether events at the outer membrane can affect the mitochondrial late pathway is not clear since Ang II has been shown to affect stimulation of the late pathway. In addition, ACTH has been shown to reduce K+ efflux from glomerulosa cells, and conceivably this action by ACTH was inhibited by pinacidil.

Additional experiments were carried out to examine the influence of pinacidil on Ang II receptor binding. Since Ang II appears to reduce K+ efflux from glomerulosa cells, we considered the possibility that pinacidil increased K+ efflux by blocking Ang II receptor binding. However, no effect on Ang II binding by pinacidil was observed. Additional studies ruled out an effect of pinacidil to antagonize binding to the ACTH receptor. The administration of 8-bromo-cAMP, which bypasses receptor binding, was also inhibited by pinacidil.

Pinacidil produced a modest decrease in ACTH-stimulated cortisol production by fasciculata cells—a decline of 10% and 20% with concentrations of 10^-6 and 10^-5 M pinacidil, respectively. There was no effect of pinacidil on the basal production rate of cortisol. The relevance of these observations to cortisol production during treatment with pinacidil is not known. There have been no published accounts of measurements of cortisol secretion in patients treated with pinacidil.

Pinacidil was not injurious to cells, as evidenced by trypan blue exclusion analysis, nor did pinacidil result in a permanent loss of secretory function. When pinacidil was subsequently removed from the incubation media, the stimulatory response to Ang II was actually augmented, as if the pinacidil had preserved the integrity of the glomerulosa's secretory capacity.

Concentrations of pinacidil that are achieved in vivo during treatment of patients with hypertension reach values of about 0.80 µM (200 ng/ml), concentrations significantly lower than the 10 µM used to inhibit aldosterone secretion in the present study. To lower contractility of vascular smooth muscle, the in vitro physiological equivalent of pinacidil's antihypertensive effect, approximately 10 µM pinacidil was required or the same amount that inhibited aldosterone production. The observations made using the bovine adrenal, bearing in mind the limitations on adrenal responsiveness imposed by conditions associated with in vitro experiments, are thus likely to have pharmacological relevance when therapeutic doses of pinacidil are used.

Other antihypertensive agents that are thought to affect vasculature by opening K+ channels include minoxidil and cromakalim. The use of both anti-hypertensive drugs has been associated with decreases in plasma aldosterone concentrations and increases in plasma renin activity, and thus both minoxidil and cromakalim may also impair the adrenal's secretion of aldosterone. Minoxidil, in addition, may lower aldosterone levels by increasing the aldosterone metabolic clearance rate. The antihypertensive benefit derived from a lowering of aldosterone levels is not known. The experience with K+ channel openers in treating patients with primary aldosteronism has not, to our knowledge, been reported.

In summary, pinacidil inhibited basal as well as stimulated aldosterone secretion in vitro. The mechanism for the inhibition was unclear. This effect of pinacidil may at least partially explain the lowering of plasma aldosterone concentrations in hypertensive patients treated with pinacidil. The present findings suggest that pinacidil may prove particularly beneficial to hypertensive patients in whom high aldosterone levels contribute to the increase in blood pressure.

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