Effect of amiloride analogs on DOCA-salt-induced hypertension in rats

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Three recent studies have demonstrated that intracerebroventricular infusions of benzamil can prevent the onset of deoxycorticosterone acetate (DOCA)-salt-induced hypertension in rats and whether this effect correlates with an inhibition of ion transport through the known amiloride-sensitive cation channels at the blood-brain barrier. We also examine whether the effects of benzamil on blood pressure are mediated by a Na⁺ channel by comparing the effects of different amiloride analogs. Benzamil (0.15 and 0.5 μg/h icv) did significantly attenuate the increase in blood pressure induced by DOCA treatment. This hypotensive effect, however, was not associated with a decrease in arterial blood pressure. In the second group of experiments, the effect of benzamil on blood pressure was examined by comparing the effects of three different amiloride analogs: benzamil, dimethyl amiloride (DMA), and 3,4-dichlorobenzamil (DCB), which have markedly different affinities for amiloride-sensitive Na⁺ channels (14).

MATERIALS AND METHODS

Experimental protocols. All procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan. Two sets of experiments were performed on adult male Sprague-Dawley rats weighing between 275 and 300 g. The first set examined changes in blood pressure in conscious DOCA-salt- and sham-treated rats 2 wk postoperative. The rats received intracerebroventriculatry either 300 mM mannitol or 300 mM mannitol with 1 mg/ml benzamil for the 2 wk. Six rats were used for each group. At the end of the infusion period, the rats were used to examine cerebrospinal fluid (CSF) K⁺ homeostasis and blood-to-brain exchange. In the second set of experiments, the effect of intracerebroventricular infusions of different concentrations (0.1, 0.3, and 1.0 mg/ml) of either benzamil, DMA, or DCB on the blood pressure was examined in conscious DOCA-salt- and sham-treated rats. Five to six rats were used for each dose. Whether the effects of the highest dose (1.0 mg/ml) of DMA and DCB on blood pressure were mediated by an inhibition of Na⁺/Ca²⁺ exchange was investigated by infusing the same dose subcutaneously. Four rats were used in each of these groups.

Animal preparation and blood pressure measurements. Rats were initially trained for tail-cuff systolic blood pressure measurement on at least three separate occasions to establish a baseline blood pressure. Systolic pressures were then measured every 3–4 days during the study.

After training for the blood pressure measurements, animals were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) intramuscularly. All animals were uninephrectomized via a flank incision with care being taken to preserve the adrenal gland and its circulation. The animals designated as DOCA-salt also received Silastic implants impregnated with DOCA (200 mg/kg body wt), which were placed subcutaneously in the dorsal neck region. These animals were given the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

0363-6135/99 $5.00 Copyright © 1999 the American Physiological Society H2215

Keep, Richard F., Xiaochen Si, Parvin Shakui, Steven R. Ennis, and A. Lorris Betz. Effect of amiloride analogs on DOCA-salt-induced hypertension in rats. Am. J. Physiol. Heart Circ. Physiol. 45: H2215–H2220, 1999.—Intracerebroventricular infusions of an amiloride analog, benzamil, reduce blood pressure in several rat models of hypertension. This effect has been attributed to an inhibition of amiloride-sensitive Na⁺ channels in the brain. This study examines whether intracerebroventricular benzamil would prevent the onset of deoxycorticosterone acetate (DOCA)-salt-induced hypertension in rats and whether this effect correlates with an inhibition of ion transport through the known amiloride-sensitive cation channels at the blood-brain barrier. We also examine whether the effects of benzamil on blood pressure are mediated by a Na⁺ channel by comparing the effects of different amiloride analogs. Benzamil (0.15 and 0.5 μg/h icv) did significantly attenuate the increase in blood pressure induced by DOCA treatment. This antihypertensive effect, however, was not associated with a decrease in arterial blood pressure. In the second group of experiments, the effect of benzamil on blood pressure was examined by comparing the effects of three different amiloride analogs: benzamil, dimethyl amiloride (DMA), and 3,4-dichlorobenzamil (DCB), which have markedly different affinities for amiloride-sensitive Na⁺ channels (14). Amiloride has effects on a number of transport systems, including Na⁺/H⁺ exchange and Na⁺/Ca²⁺ exchange (14). Benzamil is more selective than amiloride for the Na⁺ channel, but it also inhibits the other systems. By contrast, DMA preferentially inhibits Na⁺/H⁺ exchange, whereas DCB has a higher affinity for the Na⁺/Ca²⁺ exchanger and a lower affinity for the Na⁺ channel than benzamil.
Fig. 1. Changes in mean systolic blood pressure during a 2-wk intracerebroventricular infusion in sham (squares) and deoxycorticosterone acetate (DOCA)-salt (circles)-treated rats. Infusions were either 300 mM mannitol alone or mannitol + 1 mg/ml benzamil. Values are means ± SE; n = 5–6 rats. ***Significant difference between the DOCA-salt + mannitol group and each of the other groups at P < 0.001 level. †Significant difference between DOCA-salt + benzamil group and the sham + mannitol group at P < 0.05 level. There were no significant differences between sham groups infused with mannitol and benzamil.

minipumps were not attached to a lateral ventricle catheter. Instead, the pumps delivered the amiloride analogs subcutaneously (in the dorsal neck region).

Plasma and CSF electrolytes and blood-to-brain $^{22}\text{Na}$ transport. After blood pressure was monitored for 2 wk, the first set of rats was anesthetized with pentobarbital (50 mg/kg ip), and two femoral artery and one femoral vein catheters were inserted. One femoral artery catheter was used to measure blood gases and plasma ion concentrations. A CSF sample was then taken from the cisterna magna by puncture of the atlantooccipital membrane with a 26-gauge needle. Plasma and CSF Na$^+$, K$^+$, and Ca$^{2+}$ concentrations were determined by flame photometry (IL943 flame photometer, Instrumentation Laboratories, Lexington, MA) or an ion-selective electrode (GEM Premier, Mallinckrodt, Ann Arbor, MI). Blood gases and pH were determined with a blood gas analyzer (GEM Premier).

The rats were then used to measure blood-to-brain and blood-to-CSF $^{22}\text{Na}$ transport. The permeability of the BBB barrier to $^{22}\text{Na}$ was assessed by a modification of the method proposed by Ohno et al. (19) for a single-time-point analysis as previously described (2). A bolus of 0.2 ml of saline containing 15 µCi of $^{22}\text{Na}$ was injected intravenously 10 min before decapitation of the rats. Just before the $^{22}\text{Na}$ injection, an arterial blood sample was continuously withdrawn at a constant rate to determine the integral of plasma radioactivity. For the simultaneous determination of plasma volume, an additional 0.2-ml bolus of saline containing 10 µCi of $^3\text{H}$ inulin was given 2 min before decapitation of the rats. At the end of the experiment, CSF (cisterna magna) and terminal plasma samples were obtained, the rat was then decapitated, the brain was removed, and the cortices were dissected. A frontal cortex sample predominantly containing gray matter was weighed and dissolved in 1 M methylbenzethonium hydroxide at 60°C before the addition of scintillation cocktail and counting with a Beckman LS 3801 Liquid Scintillation Spectrophotometer (Fullerton, CA). Whole blood samples were similarly incubated (20 min) and then bleached with 50 µl of 30% H$_2$O$_2$ before the addition of scintillation fluid, whereas plasma and CSF samples were immediately diluted with methylbenzethonium hydroxide and scintillation cocktail.

The permeability of the BBB to $^{22}\text{Na}$ was calculated utilizing a two-compartment model for blood-to-brain transfer (19). This model assumes that tracer entry into the brain is proportional to the plasma concentration of the tracer, that there is no appreciable backflux within the period of tracer circulation, and that isotope exchange between CSF and brain extracellular space is negligible. These conditions are accomplished for the present experiments given the remote location of the tissue sample sites with regard to the ventricles by sampling only the gray matter of the frontal cortex and the shortness of tracer circulation (20). Results are

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Mean Systolic Blood Pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
</tr>
<tr>
<td>4</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>140</td>
</tr>
<tr>
<td>8</td>
<td>150</td>
</tr>
<tr>
<td>10</td>
<td>160</td>
</tr>
<tr>
<td>12</td>
<td>170</td>
</tr>
</tbody>
</table>

Table 1. Physiological parameters

<table>
<thead>
<tr>
<th></th>
<th>Weight, g</th>
<th>pH</th>
<th>$P_{CO_2}$ (mmHg)</th>
<th>$P_{O_2}$ (mmHg)</th>
<th>Hematocrit, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + mannitol</td>
<td>373 ± 12</td>
<td>7.38 ± 0.02</td>
<td>52 ± 2</td>
<td>74 ± 5</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>Sham + benzamil</td>
<td>409 ± 17</td>
<td>7.35 ± 0.01</td>
<td>65 ± 3</td>
<td>86 ± 5</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>DOCA + mannitol</td>
<td>390 ± 10</td>
<td>7.45 ± 0.02*</td>
<td>54 ± 2</td>
<td>81 ± 4</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>DOCA + benzamil</td>
<td>374 ± 11</td>
<td>7.45 ± 0.02</td>
<td>51 ± 1</td>
<td>98 ± 4</td>
<td>42 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 5–6. DOCA, deoxycorticosterone acetate. * and †Significant differences between sham and DOCA-salt-treated rats at the P < 0.05 and P < 0.01 levels. ‡Significant difference between benzamil-infused DOCA-salt and mannitol-infused sham and DOCA-salt-treated rats at the P < 0.05 level.
Expressed as an influx rate constant ($K_i$) for brain uptake, calculated as:

$$K_i = C_{iv}/\int C_{a} \, dt$$

where $C_{iv}$ is the concentration of extravascular tracer in the brain, and $\int C_{a} \, dt$ is the integral of the arterial tracer concentration. $C_{iv}$ was calculated from total tracer counts ($C_{tot}$) in the brain samples, final tracer plasma concentration ($C_{pl}$) and plasma volume (PV) as:

$$C_{iv} = C_{tot} - (PV \times C_{pl})$$

PV was determined as the [3H]inulin space of the brain samples from the [3H]inulin concentration in the final plasma sample.

**Materials.** Rats were purchased from Charles River Laboratories (Portage, MI). Benzamil was obtained from Research Biochemicals International (Natick, MA), and dimethyl amiloride was from Sigma (St. Louis, MO). 3,4-Dichlorobenzamil was provided by Research Biochemicals International as part of a Chemical Synthesis Program of the National Institute of Mental Health (Contract N01MH30003). $^{22}$Na and [3H]inulin came from New England Nuclear (Boston, MA).

**Statistical analysis.** The data are expressed as means ± SE. Statistical differences between groups were evaluated by ANOVA with a Newman-Keuls multiple comparison test. The effect of different amiloride analogs on blood pressure in sham-operated and DOCA-salt-treated rats was examined by ANOVA with a Dunnett's post hoc test.

**RESULTS**

Effects of intracerebroventricular benzamil on DOCA-salt-induced hypertension. As expected, DOCA-salt treatment caused a marked increase in blood pressure over 2 wk (Fig. 1). At 2 wk mean systolic blood pressures were elevated by ~50 mmHg compared with controls ($P < 0.001$). Intracerebroventricular infusion of benzamil (1 mg/ml at 0.5 µl/h) had no effect on blood pressure in the sham-operated rats, but it significantly blunted the increase in blood pressure found in DOCA-salt-treated rats ($P < 0.001$; Fig. 1).

At the end of the 2-wk period, there was no significant difference in body weight between sham-operated and DOCA-salt-treated rats with or without benzamil (Table 1). Similarly, there were no significant differences among the four groups in blood PCO$_2$ or hematocrit. DOCA-salt-treated rats, with or without benzamil, were alkaliotic compared with sham-operated rats. There were also some minor variations in PO$_2$ among the four groups, but these are likely to have no physiological significance.

The $K_i$ values for blood-to-brain and blood-to-CSF $^{22}$Na transport were not significantly different in sham-operated and DOCA-salt-treated rats (Fig. 2). Intracerebroventricular benzamil (1 mg/ml) also did not significantly affect the $K_i$.

Plasma and CSF [Na$^+$] were both unaffected by DOCA-salt treatment (2 wk), and intracerebroventricular benzamil did not alter either parameter in sham- or DOCA-salt-treated rats (Fig. 3A). Compared with sham-operated rats, DOCA-salt-treated rats were hypokalemic (Fig. 3B). The degree of hypokalemia was slightly less in the intracerebroventricular benzamil (1 mg/ml) group, but CSF [K$^+$] was unaffected.
Effects of amiloride analogs on DOCA-salt-induced hypertension. At a dose of 1 mg/ml (at 0.5 µl/h), the highest dose used, none of the three amiloride analogs given intracerebroventricularly had an effect on blood pressure in sham-operated rats after 2 wk (Fig. 4). In contrast, at that dose, all three analogs (benzamil, DMA, and DCB) resulted in a marked reduction in the blood pressure attained after 2 wk in DOCA-salt-treated rats (Fig. 4). The degree of reduction did not vary among the three analogs. At a lower dose (0.3 mg/ml), only DCB (P < 0.01) and benzamil (P < 0.05) significantly attenuated the rise in blood pressure resulting from DOCA-salt treatment (Fig. 4). At the lowest dose used (0.1 mg/ml at 0.5 µl/h), none of the amiloride analogs significantly inhibited the increase in blood pressure induced by DOCA-salt treatment.

Subcutaneous infusion of 1 mg/ml (at 0.5 µl/h) of DMA or DCB failed to affect blood pressure in DOCA-salt-treated rats. Thus, after 2 wk, the blood pressure in DOCA-salt-treated rats receiving 1 mg/ml of DMA was 174 ± 16 mmHg, in rats receiving 1 mg/ml of DCB was 169 ± 11 mmHg, and in rats receiving no amiloride analog was 161 ± 4 mmHg. There were no significant differences among the three groups.

Table 2 displays measurements of plasma and CSF ion concentrations after 2 wk of intracerebroventricular infusion of the highest dose of the amiloride analogs (1 mg/ml) compared with a vehicle infusion. Neither DMA nor DCB affected blood or CSF ion concentrations in sham-operated rats. Similarly, neither of these analogs affected ion concentrations in DOCA-salt-treated rats apart from a slight enhancement of the alkalosis generated by DOCA-salt treatment following intracerebroventricular infusion of DMA.

DISCUSSION

This study confirms the effect of intracerebroventricular benzamil on DOCA-salt-induced hypertension. However, this study found no evidence that intracerebroventricular benzamil affected BBB Na⁺ transport or brain K⁺ homeostasis, suggesting that it does not act by affecting the known amiloride-sensitive cation channel at the BBB. Although this result does not indicate whether intracerebroventricular benzamil might inhibit a parenchymal cell Na⁺ channel, a comparison of the effects of different amiloride analogs with those of benzamil suggests that the antihypertensive effects are not mediated by inhibiting such a channel.

Effects of intracerebroventricular benzamil on DOCA-salt-induced hypertension. Gomez-Sanchez and Gomez-Sanchez (7, 8) first reported that intracerebroventricular infusions of benzamil could prevent mineralocorticoid-induced hypertension and salt-induced hypertension in Dahl salt-sensitive rats. Nishimura et al. (18) then expanded this work, demonstrating that intracerebroventricular benzamil could reduce blood pressure in a number of forms of Na⁺-induced hypertension, including DOCA-salt-induced hypertension. These blood pressure effects of benzamil were mediated centrally because systemic administration at the doses used had no effect on blood pressure (7, 8, 18).

In the current study, we have also shown that intracerebroventricular benzamil can prevent the effects of DOCA-salt treatment on blood pressure. These

Table 2. Ion concentrations in sham and DOCA-salt rats with and without intracerebroventricular administration of amiloride analogs

<table>
<thead>
<tr>
<th></th>
<th>Blood pH</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7.41 ± 0.01</td>
<td>143 ± 1</td>
<td>152 ± 1</td>
<td>3.71 ± 0.10</td>
<td>2.73 ± 0.04</td>
<td>1.05 ± 0.09</td>
<td>0.96 ± 0.02</td>
</tr>
<tr>
<td>Sham + DMA</td>
<td>7.41 ± 0.02</td>
<td>145 ± 1</td>
<td>152 ± 1</td>
<td>3.74 ± 0.08</td>
<td>2.76 ± 0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sham + DCB</td>
<td>7.42 ± 0.01</td>
<td>141 ± 0</td>
<td>149 ± 1</td>
<td>3.36 ± 0.07</td>
<td>2.62 ± 0.01</td>
<td>1.17 ± 0.04</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td>DOCA-salt</td>
<td>7.47 ± 0.01</td>
<td>145 ± 1</td>
<td>153 ± 1</td>
<td>2.37 ± 0.07</td>
<td>2.65 ± 0.02</td>
<td>1.01 ± 0.04</td>
<td>0.89 ± 0.03</td>
</tr>
<tr>
<td>DOCA-salt + DMA</td>
<td>7.53 ± 0.01</td>
<td>142 ± 1</td>
<td>149 ± 1</td>
<td>2.36 ± 0.13</td>
<td>2.56 ± 0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DOCA-salt + DCB</td>
<td>7.51 ± 0.01</td>
<td>143 ± 1</td>
<td>152 ± 3</td>
<td>2.53 ± 0.15</td>
<td>2.58 ± 0.06</td>
<td>1.09 ± 0.04</td>
<td>1.01 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 16 rats for sham group and 23 rats for the DOCA-salt group and 5–6 rats for each other group except for [Ca²⁺], where n = 5 rats for all groups except the DOCA-salt-treated rats where n = 10. Amiloride analogs were given at 1 mg/ml. Ion concentrations in plasma (pl) and CSF are in mM. ND, not determined. * Difference between sham-operated and DOCA-salt-treated rats at the P < 0.001 level; † significant difference between an amiloride analog- and vehicle-treated group.
results complement those of Nishimura et al. (18), who found that benzamil could reduce blood pressure in DOCA-salt-treated rats with established hypertension. The doses of benzamil that were effective in our study (0.3 and 1 mg/ml infused at 0.5 µl/h) were the same as those used by Gomez-Sanchez and Gomez-Sanchez in their studies (7, 8). The method used in this study to measure conscious systolic blood pressure (the tail cuff) can have limitations in studies where blood pressure changes are small. However, the effect of DOCA-salt treatment on blood pressure and the effect of amiloride analogs on that change are of a considerable magnitude (30–50 mmHg at 14 days), negating the need for measurements with indwelling catheters.

We next examined whether intracerebroventricular benzamil at these concentrations could affect the known amiloride-sensitive cation channel at the BBB (26). This channel is a nonselective cation channel (26), and although it appears to be involved in the movement of Na+ from blood to the brain (6), it may also be involved in K+ transport at the BBB (12). Infusing benzamil at 1 mg/ml at 0.5 µl/h failed to alter either blood-to-brain 22Na transport or CSF K+ concentration, even though it did ameliorate the effects of DOCA-salt treatment on blood pressure, indicating that these antihypertensive effects are not mediated by the inhibition of the BBB ion channel.

In addition, although intracerebroventricular infusions of hypertonic NaCl (1, 25) induce increases in blood pressure, and changes in CSF Na+ concentration have been implicated in the genesis of Na+-dependent hypertension (17), we found no effect of benzamil (or any of the amiloride analogs) on CSF Na+. Indeed, in common with the findings of Takata et al. (23) and Nishimura et al. (18), we found no effect of DOCA-salt treatment on CSF Na+ concentration.

Effects of amiloride analogs. The absence of an effect of benzamil on BBB Na+ transport (and CSF Na+ and K+ concentration) suggests that benzamil does not have its antihypertensive effect by inhibiting the known BBB nonselective cation channel. A comparison of the effects of the different amiloride analogs on DOCA-salt-induced hypertension suggests that the antihypertensive effects are not mediated by the inhibition of a parenchymal cell Na+ channel but may rather be via an alteration in brain Ca2+ homeostasis. As with benzamil (7, 8, 18), the blood pressure effects of DMA and DCB appear to be mediated centrally because systemic administration did not affect blood pressure in DOCA-salt-treated rats.

Amloride has effects on a wide range of ion transporters and ion channels, including Na+/H+ exchange and certain Na+ channels (reviewed in Ref. 14). Specific amiloride analogs have been developed that have different affinities for these transporters. Thus benzamil inhibits amiloride-sensitive Na+ channels at concentrations 10 times lower than with amiloride and 100 times lower than with DMA (14). In contrast, DMA inhibits Na+/H+ exchange at concentrations 10 times lower than with amiloride and 100 times lower than with benzamil (14). The fact that benzamil and DMA have potencies for the Na+ channel and the Na+/H+ exchanger that differ by two orders of magnitude but have almost the same potency in inhibiting the hypertensive effect of DOCA-salt treatment suggests that this antihypertensive effect is not mediated through a Na+ channel or Na+/H+ exchange.

Amiloride and its analogs are also inhibitors of Na+/K+-ATPase and Na+-coupled transport (e.g., Na+/PO43− transport). However, these actions are unlikely to be involved in the antihypertensive actions described in this study. Inhibition of these types of transport requires concentrations close to the millimolar range (14), and the concentration in CSF in the present experiments may, at the highest dose, be closer to 10–15 µM [rat CSF production of 2 µl/min (11) would be expected to dilute the infusate 240-fold]. In addition, central administration of a Na+/K+-ATPase inhibitor ouabain actually causes increases in blood pressure (9).

Amiloride analogs also inhibit Na+/Ca2+ exchange (14). Unlike potencies for the Na+ channel and the Na+/H+ exchanger, the potencies of DMA and benzamil for the Na+/Ca2+ exchanger differ by less than an order of magnitude with benzamil having the higher affinity. DCB has a higher affinity for the Na+/Ca2+ exchanger than either of the other amiloride analogs, but its potency is still only about 10 times that of DMA. Thus, unlike with the Na+ channel and the Na+/H+ exchanger, the relative potency of the different amiloride analogs for inhibiting Na+/Ca2+ exchange fairly closely matches their antihypertensive effects. In vitro with astrocytes and brain membrane preparations, DCB inhibits Na+/Ca2+ exchange with an IC50 of about 15–30 µM (10, 24). These values are similar to the CSF concentrations expected from the intracerebroventricular infusions used in the experiments in this study.

There have been numerous studies in humans and animals examining the role of Ca2+ in the regulation of blood pressure (reviewed in Ref. 21) and intracerebroventricular infusions of Ca2+ lower blood pressure (4, 15, 22). Amloride analog-induced inhibition of Na+/Ca2+ exchange might be expected to have a similar effect on blood pressure by raising intracellular Ca2+ concentrations. Amloride analogs can also inhibit Ca2+ channels (3, 14), but such inhibition would be expected to lower intracellular Ca2+ concentrations.

In summary, these results support previous findings on the antihypertensive effects of intracerebroventricular amiloride but suggest that these are not mediated by the inhibition of brain Na+ channels. Rather, the intracerebroventricular administration of amiloride analogs may ameliorate the effects of DOCA-salt treatment on blood pressure by inhibiting Na+/Ca2+ exchange.

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