Calcium and Prostaglandin E$_2$ in Renomedullary Interstitial Cells

Patricia A. Craven and Frederick R. DeRubertis

Renomedullary interstitial cells cultured from the Dahl salt-resistant rat have higher levels of basal cytosolic calcium and prostaglandin E$_2$ and are more responsive to vasopressin than interstitial cells from the Dahl salt-sensitive rat. We examined the potential role of inositol phospholipid hydrolysis in mediating these differences. Vasopressin-induced increases in labeled inositol phosphates were enhanced in renomedullary interstitial cells from Dahl salt-resistant compared with those from salt-sensitive rats. Addition of neomycin reduced basal production of labeled inositol phosphates and abolished the increase in inositol phosphates induced by vasopressin. Neomycin also prevented the peak decline pattern in cytosolic Ca$^{2+}$ seen with vasopressin but did not influence basal cytosolic Ca$^{2+}$. In the presence of neomycin, vasopressin induced a modest but prolonged increase in cytosolic calcium. In contrast to its marked effects on inositol phosphate production, neomycin was without effect on basal or vasopressin-responsive prostaglandin E$_2$ production. Moreover, basal and vasopressin-induced increases in cytosolic Ca$^{2+}$ remained higher in renomedullary interstitial cells from Dahl salt-resistant versus those from salt-sensitive rats exposed to neomycin. The results do not support a requirement for phospholipase C-induced inositol phospholipid hydrolysis in the mediation of vasopressin actions on prostaglandin E$_2$ production by renomedullary interstitial cells and imply that the differences in cytosolic Ca$^{2+}$ and prostaglandin E$_2$ seen in these two cell lines are not related to differences in inositol phospholipid metabolism. (Hypertension 1991;17:303–307)

We previously demonstrated that basal and arginine vasopressin (AVP)–induced increases in cytosolic Ca$^{2+}$ (Ca$^{2+}$i) are enhanced in renomedullary interstitial cells (RMIC) prepared from Dahl salt-resistant (DR) versus salt-sensitive (DS) rats. Differences were expressed in cells cultured from prehypertensive DS rats and persisted through at least 20 passages, suggesting a genetic basis. The higher levels of Ca$^{2+}$i in RMIC from DR rats were associated with higher basal and AVP-responsive arachidonate release and prostaglandin (PG) E$_2$ production by these cells. In contrast, arachidonate-induced increases in PGE$_2$ production were not different in the two cell lines.

Neomycin binds specifically to phosphatidylinositol 4,5-bisphosphate and has been used extensively as an inhibitor of inositol phospholipid metabolism and phospholipase C. In the present study, we used neomycin to examine the potential role of altered inositol phospholipid turnover in the mediation of the differences in Ca$^{2+}$i and PGE$_2$ observed. The results dissociate AVP-induced increases in inositol phospholipid hydrolysis from PGE$_2$ production and demonstrate that the higher levels of basal and AVP-responsive Ca$^{2+}$i and PGE$_2$ observed in RMIC from DR versus DS rats are not explained by enhanced receptor-mediated inositol phospholipid hydrolysis.

Methods

Treatment of Rats

Male DS and DR rats were obtained from Brookhaven National Laboratories, Upton, N.Y., at weaning (3 weeks of age) and were maintained on a low sodium (0.07–0.09% Na$^+$) diet until 6 weeks of age. Indirect tail-cuff measurements of blood pressure were obtained in conscious rats before they were killed. Four readings were obtained per rat.

Culture of Renomedullary Interstitial Cells

RMIC were prepared from 6-week-old DS and DR rats for culture as previously described. Light microscopy of cultured RMIC, which had been stained with oil red O, demonstrated numerous lipid-containing cytoplasmic vesicles, which are character-
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Incubation of Cells and Assay of Prostaglandin E₂

Hypertension

Determination of Cytosolic Ca²⁺

KRBG

Ca²⁺-free KRBG

Table 1. Effects of Brief or Prolonged Exclusion of Media Calcium on Basal and Arginine Vasopressin-Responsive Prostaglandin E₂ and Cytosolic Ca²⁺ in Renomedullary Interstitial Cells From Dahl Salt-Sensitive and Salt-Resistant Rats

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ca²⁺, (nM)</th>
<th>PGE₂ (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DS</td>
<td>DR</td>
</tr>
<tr>
<td>Preincubation I</td>
<td>-AVP</td>
<td>+AVP</td>
</tr>
<tr>
<td>(55 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRBG</td>
<td>107±9</td>
<td>350±28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRBG</td>
<td>102±8</td>
<td>359±48</td>
</tr>
<tr>
<td>Ca²⁺-free KRBG</td>
<td>78±66</td>
<td>84±7</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Renomedullary interstitial cells were preincubated in complete or Ca²⁺-free Krebs-Ringer bicarbonate buffer with 100 mg/dl glucose (KRBG) for the times shown under incubations I and II before starting the measurements of cytosolic Ca²⁺ (Ca²⁺i) and prostaglandin E₂ (PGE₂). Where indicated, arginine vasopressin (AVP) was present at 10⁻⁷ M. DS, Dahl salt-sensitive rats; DR, Dahl salt-resistant rats.

*p<0.05 compared with value obtained in complete KRBG.

Calcium concentrations were determined by atomic absorption spectrometry.

Results were obtained by sequential perfusion of media solutions versus the negative log of the observed fractional luminescence. Fractional luminescence was determined by dividing the observed luminescence by the maximal luminescence obtained when cells were perfused with 1% Triton X-100.

Determination of Inositol Phosphate Production

RMIC were grown to confluence in Eagle's basal medium plus glutamine, penicillin, and streptomycin. All incubations for PGE₂ determination were conducted with confluent cultures in 24-well plates at 37°C. The gas phase was 7% CO₂, balance air. RMIC were preincubated under the conditions indicated in the tables. The media was then changed to fresh media of the same composition, and media PGE₂ accumulation was determined during a subsequent 30-min incubation period. When present, AVP (10⁻⁷ M) was added during the 30-min incubation period for determination of PGE₂. To determine the effect on basal and AVP-responsive PGE₂ of removal of extracellular Ca²⁺ from the incubation medium (Table 1), RMIC were exposed to Ca²⁺-free Krebs-Ringer bicarbonate buffer with 100 mg/dl glucose (KRBG) for either 5 or 60 minutes before changing to fresh media of the same concentration and beginning the 30-min incubation for measurement of media PGE₂. The times of exposure to Ca²⁺-free KRBG (5 or 60 minutes) were chosen based on preliminary experiments, which showed that removal of extracellular calcium for 5 minutes had no effect on AVP-responsive Ca²⁺, whereas removal of extracellular calcium for 60 minutes prevented the AVP-induced increase in Ca²⁺.

PGE₂ content of the final incubation media was determined by radioimmunoassay (Du Pont, New England Nuclear, Boston).

Calcium concentrations were determined by atomic absorption spectrometry.

The procedure for loading aequorin was as previously described. Aequorin was purchased from Dr. J.R. Blinks (Mayo Foundation, Rochester, Minn.). RMIC, which had been incorporated into agarose threads, were suspended in an aequorin photometer and were perfused with KRBG 95% O₂-5% CO₂ mixture at 1 ml/min for the times and with test agents as indicated in the text. The sample compartment was maintained at 37°C. Using this protocol, multiple determinations of Ca²⁺, could be made with the same cell preparation under different experimental conditions. RMIC were preincubated under the conditions shown in the tables before obtaining measurements of Ca²⁺. Results were obtained by sequential perfusion of the same cell preparation under the conditions shown. Unless otherwise indicated, peak responses to AVP are given. Cells were perfused with complete KRBG 95% O₂-5% CO₂ mixture in the absence of AVP for 30 minutes after each period of stimulation with AVP. Ca²⁺, was derived from a curve of the negative log of [Ca²⁺] in standard solutions versus the negative log of the observed fractional luminescence. Fractional luminescence was determined by dividing the observed luminescence by the maximal luminescence obtained when cells were perfused with 1% Triton X-100.

Determination of Inositol Phosphate Production

RMIC were grown to confluence in a T-75 flask and labeled for 3 days with [³H]inositol (5 μCi/10 ml) in inositol-free medium. Cells were then detached with trypsin, washed with serum-free medium, resuspended in serum-free medium plus 20 mM LiCl, and aliquoted (1 x 10⁶ cells/0.5 ml/tube). RMIC were preincubated with or without 1.5 mM neomycin for 30 minutes. AVP (10⁻⁷ M) was then added to some of the flasks. Reactions were stopped after 1 minute with 0.67 ml of 10% HClO₄ and labeled inositol phosphates were isolated as previously described. In some experiments, neomycin was added to the flasks at the end of the AVP incubation period to verify that neomycin did not interfere with the inosi-
Results

Table 1 compares the effects of a brief or prolonged period of media calcium deprivation on basal and AVP-induced increases in PGE$_2$ and Ca$^{2+}$ in DS and DR rats. Blood pressure in both groups of rats, as determined by the tail-cuff method, was normal (DS rats, 105±4 mm Hg; DR rats, 108±3 mm Hg). Consistent with previous results, basal and AVP-induced increases in Ca$^{2+}$ and PGE$_2$ were higher in RMIC from DR versus DS rats. As is shown, removal of calcium from the medium for 5 minutes had no effect on basal Ca$^{2+}$ or on the subsequent peak response to AVP. Basal and AVP-induced increases in the accumulation of PGE$_2$ during a subsequent 30-minute incubation period in the continual absence of media calcium were also not affected by a brief 5-minute preincubation in the absence of calcium. By contrast, preincubation of RMIC for 60 minutes also reduced basal PGE$_2$ production and abolished responses to AVP when PGE$_2$ was assessed during the subsequent 30-minute incubation period in the continual absence of calcium. As is also shown in Table 1, basal Ca$^{2+}$ and PGE$_2$ remained significantly higher in interstitial cells from DR versus DS rats even when the cells were exposed to Ca$^{2+}$-free media for 60 minutes.

Table 2 illustrates the effects of neomycin on basal and AVP-induced increases in labeled inositol phosphate production by RMIC from DS and DR rats. The time of exposure to neomycin and the concentration used were chosen based on the results of preliminary measurements of Ca$^{2+}$, which demonstrated that exposure of RMIC to 100 or 500 mM neomycin for 30 minutes had no effect on AVP-induced increases in [Ca$^{2+}$], whereas 1.5 mM neomycin blocked the peak decline in [Ca$^{2+}$], seen with AVP. This concentration of neomycin had no effect on cell viability, as demonstrated by trypan blue exclusion and an intact PGE$_2$ response to AVP (Table 3). As is shown in Table 2, basal inositol phosphate production was not significantly different in RMIC from DR versus DS rats. However, the increase in response to AVP was significantly greater in RMIC from DR compared with DS rats. As is also shown in Table 2, exposure of RMIC to 1.5 mM neomycin reduced basal inositol phosphate production and abolished responses to AVP in both groups. In other experiments (not shown), AVP-induced increases in inositol phosphate production were also suppressed by neomycin when the time of incubation with AVP was increased to 15 minutes or the concentration of AVP used was raised to $10^{-6}$ M.

Tables 3 and 4 illustrate the effects of neomycin on basal and AVP-induced increases in PGE$_2$ and Ca$^{2+}$ in RMIC from DS and DR rats. Ca$^{2+}$ values shown are 1) the basal value, which remained constant for 30 minutes before addition of AVP; 2) the peak response after addition of AVP; and 3) the value obtained 15 minutes after AVP stimulation. As shown in Table 3, exposure of RMIC to neomy-

<table>
<thead>
<tr>
<th>Solution</th>
<th>-AVP (ng/mg protein)</th>
<th>+AVP 10$^{-7}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRBG</td>
<td>23±3</td>
<td>88±12</td>
</tr>
<tr>
<td>+1.5 mM neomycin</td>
<td>29±3</td>
<td>77±9</td>
</tr>
<tr>
<td>Values are for prostaglandin E$_2$ (PGE$_2$) (ng/mg protein). Renomedullary interstitial cells were preincubated for 30 minutes under the conditions shown before starting the measurement of PGE$_2$. DS, Dahl salt-sensitive rats; DR, Dahl salt-resistant rats; AVP, arginine vasopressin; KRBG, Krebs-Ringer bicarbonate buffer with 100 mg/dl glucose.</td>
<td></td>
<td></td>
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</table>
cin (1.5 mM) had no effect on basal PGE$_2$ or on PGE$_2$ responses to AVP of RMIC from DS or DR rats. As is shown in Table 4, neomycin had no effect on basal Ca$^{2+}$, but suppressed the absolute AVP-induced increase in Ca$^{2+}$, by about 50%. In the presence of neomycin, a peak decline pattern in [Ca$^{2+}$], was not observed. By contrast, Ca$^{2+}$, remained significantly higher than basal for 15 minutes after addition of AVP to RMIC exposed to neomycin but had returned to basal by that time in cells not exposed to neomycin. Ca$^{2+}$, returned to baseline by 30 minutes after AVP addition in cells exposed to neomycin plus AVP (not shown).

### Discussion

AVP-induced increases in phospholipase A$_2$ activity, arachidonate release, and PGE$_2$ production are presumed to occur in response to phospholipase C-mediated inositol phospholipid hydrolysis and mobilization of intracellular calcium stores.$^{12,13}$ In the present study, AVP-induced increases in inositol phosphate production and the peak Ca$^{2+}$, response were higher in DR versus DS rats, suggesting that differences in phospholipase C-mediated inositol phosphate hydrolysis might be linked to the differences in PGE$_2$ production between these cell lines. However, recent studies have suggested that phospholipase C-mediated inositol phospholipid hydrolysis can be dissociated from and may not be a requirement for arachidonate release.$^{5,6,14,15}$ In the present study, neomycin, an agent that has previously been shown to block phosphoinositide turnover and phospholipase C action,$^{2-6}$ reduced basal and abolished AVP-induced increases in inositol phosphate production by RMIC. Neomycin also prevented the peak decline response of Ca$^{2+}$, to AVP but had no effect on basal or AVP-induced increases in PGE$_2$ production. These studies strongly suggest that phospholipase C-mediated inositol phospholipid hydrolysis is not required for maintenance of basal or AVP-responsive PGE$_2$ in RMIC. However, they do not rule out the possibility that AVP-induced increases in inositol phosphate production and mobilization of intracellular calcium contribute to the increases in PGE$_2$ production that occur in the absence of neomycin.

As demonstrated in the present study, AVP increased Ca$^{2+}$, in the presence or absence of neomycin. However, the responses differed under the two conditions. In the absence of neomycin, AVP induced a typical peak decline pattern in Ca$^{2+}$, that was not altered by a brief exclusion of perfusate calcium and therefore may have arisen from mobilization of intracellular calcium stores. By contrast, in the presence of neomycin, the AVP-induced increase in Ca$^{2+}$, was reduced by about 50%. However, the increase persisted for a longer period of time, and Ca$^{2+}$, was actually higher in the presence than in the absence of neomycin between 3 and at least 15 minutes after AVP. This increase of Ca$^{2+}$, may be involved in expression of the PGE$_2$ response to AVP.

The mechanisms that mediate the differences between RMIC from DR versus DS rats with respect to basal and AVP-responsive Ca$^{2+}$, and PGE$_2$ are not known. Some reports have described increases in phospholipid turnover in platelets$^{16-18}$ and vascular smooth muscle cells$^{19}$ from hypertensive rats or patients. In contrast, decreases in inositol phospholipid turnover have been reported in rat renal cortex$^{20}$ and erythrocytes$^{17}$ from hypertensive rats. Results of the current study in RMIC imply that factors other than phospholipase C-mediated inositol phospholipid hydrolysis are involved. Mechanisms that modulate Ca$^{2+}$, and might mediate AVP-induced increases in Ca$^{2+}$, include passive calcium diffusion, calcium pump activity, and the activity of calcium binding proteins. The extent, if any, to which these regulatory factors of Ca$^{2+}$, differ in RMIC from DR versus DS rats remains to be established.

### Acknowledgments

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### References


#### Table 4. Effects of Neomycin on Basal and Arginine Vasopressin-Induced Increases in Cytosolic Ca$^{2+}$ in Renomedullary Interstitial Cells From Dahl Salt-Sensitive and Salt-Resistant Rats

<table>
<thead>
<tr>
<th>Solution</th>
<th>DS -AVP (nM)</th>
<th>Peak (nM)</th>
<th>15 min (nM)</th>
<th>DR -AVP (nM)</th>
<th>Peak (nM)</th>
<th>15 min (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRBG</td>
<td>109±8</td>
<td>362±23</td>
<td>112±6</td>
<td>205±18</td>
<td>756±71</td>
<td>214±23</td>
</tr>
<tr>
<td>+1.5 mM neomycin</td>
<td>115±5</td>
<td>216±21*</td>
<td>183±20*</td>
<td>214±17</td>
<td>434±39*</td>
<td>397±42*</td>
</tr>
</tbody>
</table>

Renomedullary interstitial cells were preincubated for 30 minutes under the conditions shown before addition of arginine vasopressin (AVP). Peak cytosolic Ca$^{2+}$ (Ca$^{2+}$,) response after AVP addition is shown as well as the level of Ca$^{2+}$, 15 minutes after addition of AVP. DS, Dahl salt-sensitive rats; DR, Dahl salt-resistant rats; KRBG, Krebs-Ringer bicarbonate buffer with 100 mg/dl glucose.

$^*p<0.05$ compared with corresponding value in the absence of neomycin.


KEY WORDS • calcium • prostaglandins • renal function • neomycin • inositol phosphates
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