Regulation by PKC isoforms of Na\(^+\)/H\(^+\) exchanger in luminal membrane vesicles isolated from cortical tubules

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FOUR ISOFORMS of Na/H antiporters have been described in the kidney and designated as NHE1 to NHE4. NHE1 and NHE4 are located in the basolateral membrane of the renal tubule (6, 8), and NHE3 and NHE2 are apical isoforms (5, 9). NHE3 has been detected in rat proximal tubule by in situ hybridization approach (12). Hoechst 694 (Hoe-694, 100 µM), which completely inhibits NHE2 transport activity and only marginally NHE3 activity, has no effect on HCO3\(^-\) absorption in the proximal tubule in vivo (26). Finally, in mice lacking NHE3, the degree of inhibition of HCO3\(^-\) absorption in perfused proximal tubule in vivo corresponds to the total activity of the apical Na/H exchanger (24). Second, it is possible that the renal tubule provides a unique environment leading to tissue-specific regulation of NHE3 related, for example, to different PKC isoform profiles or differential activation of isoforms in the proximal tubule and cultured cells.

Angiotensin II (ANG II), which has a well-known dose-dependent biphasic effect on the proximal luminal Na/H exchange activity, induces a concentration-dependent increase in inositol trisphosphate (IP\(_3\)) production and cytosolic Ca\(^{2+}\), which implies a dose-dependent increase in phospholipase C activity (22). In rat cortical tubule suspension, low-dose ANG II (10\(^{-11}\) M) stimulates the luminal Na/H exchange through a PKC-dependent pathway (14). In contrast, high-dose ANG II (10\(^{-7}\) M) inhibits the luminal Na/H exchange activity through phospholipase A\(_2\) (PLA\(_2\)) and cytochrome P-450-dependent metabolites of arachidonate, likely 5,6-epoxyeicosatrienoic acid, and the inhibition of the latter pathway unmasks the stimulatory effect of ANG II (14). Whether the stimulation of specific PKC isoforms by high-dose ANG II might contribute to the stimulation of PLA\(_2\) is not known (14).

Therefore the aim of the present study was to determine the effects of acute phorbol ester and ANG II pretreatment of the cortical tubule suspension on the Na/H activity and abundance of PKC isoforms in LMV.
MATERIALS AND METHODS

Preparation of partially purified LMV. A suspension of cortical tubules free of glomeruli was prepared as previously described (22). Five-milliliter samples of tubule suspension in a Ringer medium (in mM: 116 NaCl, 3 KCl, 1 MgSO 4, 0.2 KH 2PO 4, 0.8 K 2HPO 4, 10 HEPEs, 1 CaCl 2, 25 NaHCO 3, 5 glucose, 5 alanine, 10 sodium pyruvate, and 0.1% bovine serum albumin) were equilibrated at 37°C under an atmosphere of 95% O 2-5% CO 2 for 15 min before the addition of the agent that had to be tested. The incubation was stopped by adding 10 ml of ice-cold Ringer medium. After centrifugation, the tubules were resuspended in hypsomotic homogenization medium (in mM: 125 mannitol, 2 dithiothreitol, 5 Trizma, pH 7.4, 10 EGTA-Tris, pH 7.4, 10 benzamidine, 0.2 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] with 0.1 µg/ml aprotinin and 12.5 µg/ml leupeptin, 180 mosmol/kgH 2O. Purified LMV were prepared by Mg 2+ precipitation as described in (4). The final pellet was resuspended at 2–4 µg/ml in homogenization medium for Western blot experiments. For 22Na uptake experiments, LMV were resuspended in a Na-free medium (in mM: 200 mannitol, 3 EGTA, 50 betamethylammonium (TMA) nitrate, and 50 Tris-MES, pH 6), pelleted (30,000 g for 30 min, 4°C), resuspended in the same acidic medium, and kept at −80°C until use. The purity and yield of the LMV preparation were routinely followed by measuring the activity of enzyme markers maltase (10) and Na + -K + -ATPase (11). There was no difference between the membranes prepared from control tubules and those pretreated with phorbol esters or ANG II, regarding the specific activity of the markers (not shown) as well as yield (33 ± 3 and 32 ± 3% for maltase, and 6 ± 1 and 6 ± 1% for Na + -K + -ATPase of controls and phorbol myristate acetate (PMA), respectively; 44 ± 3 and 43 ± 3% for maltase, and 4.9 ± 0.5 and 5.3 ± 0.6% for Na + -K + -ATPase of controls and ANG II, respectively) or enrichment (10 ± 1 and 9 ± 1 for maltase, and 1.8 ± 0.1 and 2.0 ± 0.1 for Na + -K + -ATPase of controls and PMA, respectively; and 13 ± 1 and 13 ± 1 for maltase, and 1.5 ± 0.1 and 1.7 ± 0.2 for Na + -K + -ATPase of controls and ANG II, respectively).

Immunoblot analysis. Aliquots of 90 µl of LMV fractions were mixed with 30 µl of Laemmli buffer, heated at 90°C for 10 min and stored at −20°C until use. Aliquots were subjected to SDS-PAGE (7.5%) as described by Laemmli (17); equivalent amounts of protein from controls or experimentals were run in parallel. Each sample was run in duplicate. Proteins on the gel were electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, 0.45 mm) by using a Bio-Rad apparatus. The blots were rinsed and incubated with affinity-purified anti-NHE2 or anti-NHE3 polyclonal antibodies (Santa Cruz for α, δ, and ε; Life Technologies for γ) diluted (1/1,000 for α and δ, 1/500 for ε, and 1/1,500 for γ). After 1 hr at room temperature for all antibodies used except PKC-ζ (overnight at 4°C), the nitrocellulose membranes were washed and probed with horseradish peroxidase-conjugated goat anti-rabbit antibody and then developed with an enhanced chemiluminescence kit (ECL) from Amersham. Polaroid pictures were taken with an Amersham apparatus. Apparent molecular masses were calculated on the basis of the mobility of a panel of molecular mass markers from Sigma. Quantitative data were obtained by scanning the photos (Hewlett-Packard Scanjet CX using Deskan) and analyzed with NIH image software.

RESULTS

Identification of NHE proteins and NHE transport activities in LMV isolated from cortical tubule suspensions. NHE proteins present in LMV were characterized by Western blot analysis (Fig. 1, A and B). Under basal conditions, anti-NHE3 antibodies, whose specificity was documented in a previous study (2), were used to confirm the presence of NHE3 in the LMV. Anti-NHE3 antibodies reacted with 83- to 85-kDa protein. Anti-NHE2 antibodies, whose specificity was also documented in a previous study (9), reacted with an 85-kDa protein. The presence of NHE2 protein in the preparation represents a contamination with distal cortical structures, cortical thick ascending limb and distal convoluted tubule, where NHE2 protein is present (9). No immunoreactivity was detected with anti-NHE1 antibodies (data not shown).

Na/H exchange activity in LMV was assessed by the initial rate of 22Na uptake in the presence of an outward H + gradient. In preliminary experiments with LMV, we found an IC 50 value of 10 µM EIPA, and we used 100 µM EIPA to completely inhibit the Na/H exchange activity as documented by others (21). As shown in Fig. 1C, LMV total Na uptake was markedly inhibited by 100 µM EIPA (81% inhibition) and was almost completely resistant to 100 µM Hoe-694 (7% inhibition), which was previously documented to completely inhibit NHE2 activity and marginally NHE3 (29, 26). These results supported that EIPA-sensitive Na uptake represented Na/H exchange activity due to NHE3 in LMV. However, to be sure that the small Hoe-694-sensitive Na uptake was not due to the NHE2 protein present in the preparation and located in the distal structures, we tested our ability to measure the transport activity of other transport proteins, the cotransporters BSc1 (bmetanide sensitive) and TSC (thiazide sensitive), also present in the preparation and located in cortical distal structures (9). No component of 22Na uptake inhibited by bumetanide or thiazide was observed in LMV incubated with 100 mM KCI. Actually, KCl did not elicit
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22Na uptake different from that measured in LMV incubated without KCl (data not shown), whereas we were able to measure BSC1 transport activity in basolateral membranes of medullary thick ascending limb with the same protocol (23). It should be noted that no component sensitive to 1 µM Hoe-694, i.e., NHE1 activity, was present, confirming with Western blot analysis that LMV were not contaminated with basolateral membranes. Finally, NHE4 activity, which is a basolateral isoform (8), with amiloride sensitivity close to that of NHE3, could not have contributed to the Hoe-694-resistant Na uptake.

Effects of PMA on NHE3 protein abundance and Na/H exchange activity in LMV. As shown in Fig. 1B, NHE3 protein abundance in LMV was unchanged after PMA treatment of the suspension (108.4 ± 15.1% of controls, n = 4). The values of Na uptake in LMV when cortical tubules were previously exposed to PMA (10−7 M for 4 min) are shown in Table 1. Total Na uptake was enhanced by 9.6 ± 3.8% (n = 11, P < 0.02), and the residual EIPA-resistant component of Na uptake did not change. EIPA-sensitive Na uptake increased by 12 ± 3.5% (n = 11, P < 0.001). Thus PMA increased NHE3 activity in LMV.

Preincubation of the cortical tubule suspension with specific PKC inhibitor bisindolylmaleimide (10−5 M for 5 min) followed by an additional 4-min incubation with PMA resulted in a complete inhibition of the PMA effect on EIPA-sensitive Na uptake (Table 1). EIPA-sensitive Na uptake was similar in controls and bisindolylmaleimide-pretreated tubules (not shown).

Effect of pretreatment of cortical tubules with PMA on PKC isoform abundance in LMV. As shown in Fig. 2, proteins immunoreacting with anti-PKC isoforms α, δ, ε, and ζ were detected in LMV isolated from control tubules, and the abundance of PKC isoforms α, δ, and ε was increased in LMV isolated from tubules incubated with PMA (140 ± 11, 182 ± 24, and 133 ± 8% of controls for α, δ and ε, respectively, n = 10). The inactive phorbol didecanoate had no effect (Fig. 2).

Effects of pretreatment of cortical suspensions with low-dose ANG II (10−10 M) on Na/H exchange activity and PKC isoform abundance in LMV. The values of Na uptake in LMV when cortical tubule suspensions were

Table 1. Effect of PMA on 22Na uptake

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PMA</th>
<th>Difference</th>
<th>Paired t-Test</th>
<th>PMA + Bis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 22Na uptake</td>
<td>1.71 ± 0.15</td>
<td>1.87 ± 0.18</td>
<td>0.16 ± 0.07</td>
<td>&lt;0.02</td>
<td>1.68 ± 0.24</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIPA resistant</td>
<td>0.34 ± 0.04</td>
<td>0.34 ± 0.04</td>
<td>0.00 ± 0.00</td>
<td>&lt;0.001</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIPA sensitive</td>
<td>1.38 ± 0.13</td>
<td>1.54 ± 0.15</td>
<td>0.16 ± 0.05</td>
<td>&lt;0.001</td>
<td>1.38 ± 0.20</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equilibrium</td>
<td>1.53 ± 0.13</td>
<td>1.60 ± 0.12</td>
<td>0.06 ± 0.05</td>
<td>&lt;0.001</td>
<td>1.68 ± 0.24</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of n experiments and expressed in nmol·mg protein−1·10−6 s−1 or nmol·mg protein−1·180 min−1 for values at equilibrium. Cortical tubule fragments preincubated with 10−5 M bisindolylmaleimide (PMA + Bis) or vehicle (0.1% DMSO, Control, PMA) were treated with PMA (10−7 M, 4 min) or vehicle (0.1% ethanol, Control), and LMV were subsequently isolated. H+ stimulated 22Na uptake was determined with or without EIPA (100 µM). EIPA, ethylisopropylamiloride; PMA, phorbol myristate acetate; and LMV, luminal membrane vesicles.
previously incubated with $10^{-11}$ M ANG II for 4 min are shown in Table 2. Total Na uptake increased by 10.0 ± 2.3% ($n = 7$, $P < 0.001$), and the residual EIPA-sensitive component of Na uptake did not change. EIPA-sensitive Na uptake increased by 11 ± 2.6% ($n = 7$, $P < 0.001$). Thus low-dose ANG II increased NHE3 activity. Preincubation of cortical tubules with bisindolylmaleimide resulted in a complete inhibition of the ANG II effect on EIPA-sensitive Na uptake (Table 2).

As shown in Fig. 3, when cortical tubule suspension was incubated with $10^{-11}$ M ANG II for 4 min, only PKC-δ abundance was increased in LMV (193 ± 27% of controls; $n = 4$, $P < 0.02$). The abundance of PKC isoforms α, δ, and ε remained unchanged (115 ± 11, 117 ± 12, and 88 ± 9% of controls for PKC isoforms α, δ, and ε, respectively; $n = 3$, not significant).

Effect of pretreatment of cortical tubules with high-dose ANG II on Na/H exchange activity in LMV. As shown in Fig. 4, incubation of the cortical tubule suspension with $10^{-7}$ M ANG II (10 min) decreased the EIPA-sensitive Na uptake by 13.4 ± 3.4% (P < 0.02, $n = 5$). This inhibitory effect of ANG II was observed when the tubules were preincubated with bisindolylmaleimide ($10^{-5}$ M, 5 min). Thus $10^{-7}$ M ANG II inhibited NHE3 activity in LMV, but this effect was not PKC mediated.

Table 2. Effect of low dose ANG II on $^{22}$Na uptake

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ANG II, $10^{-11}$ M</th>
<th>Difference</th>
<th>Paired t-Test</th>
<th>ANG II + Bis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total $^{22}$Na uptake</td>
<td>2.24 ± 0.30</td>
<td>2.47 ± 0.34</td>
<td>0.23 ± 0.05</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>EIPA resistant</td>
<td>0.32 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.05 ± 0.02</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>EIPA sensitive</td>
<td>1.92 ± 0.28</td>
<td>2.13 ± 0.31</td>
<td>0.20 ± 0.05</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Equilibrium</td>
<td>2.05 ± 0.31</td>
<td>2.07 ± 0.32</td>
<td>0.02 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total $^{22}$Na uptake</td>
<td>2.89 ± 0.45</td>
<td>3.25 ± 0.45</td>
<td>0.36 ± 0.03</td>
<td>&lt;0.05</td>
<td>2.91 ± 0.47</td>
</tr>
<tr>
<td>EIPA resistant</td>
<td>0.36 ± 0.04</td>
<td>0.40 ± 0.05</td>
<td>0.04 ± 0.01</td>
<td>&lt;0.05</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>EIPA sensitive</td>
<td>2.54 ± 0.42</td>
<td>2.86 ± 0.41</td>
<td>0.32 ± 0.02</td>
<td>&lt;0.05</td>
<td>2.54 ± 0.42</td>
</tr>
</tbody>
</table>

Values are means ± SE of $n$ experiments and expressed in nmol·mg protein$^{-1}$·10 s$^{-1}$ or nmol·mg protein$^{-1}$·180 min$^{-1}$ for values at equilibrium. Cortical tubule fragments preincubated with $10^{-5}$ M bisindolylmaleimide (ANG II + Bis) or vehicle (0.1% DMSO, Control, ANG II) were treated without (control) or with ANG II ($10^{-11}$ M, 4 min), and LMV were subsequently isolated. H$^+$-stimulated $^{22}$Na uptake was determined with or without 100 µM EIPA.

DISCUSSION

The present study was designed to identify the Na/H antiporter isoforms present in the luminal membranes isolated from rat kidney cortical tubules and to examine the effects of acute pretreatment with PMA or ANG II on NHE activity and PKC isoform abundance. The main informative points are the following. 1) In LMV, the Na/H exchange activity observed was attributed to NHE3. 2) PMA pretreatment increased LMV Na/H exchange activity while NHE3 protein abundance remained unchanged in LMV. PMA increased abundance of PKC isoforms α, δ, and ε in LMV. PKC inhibition suppressed the PMA effects. 3) Low-dose ANG II increased LMV Na/H exchange activity and only PKC-δ abundance in LMV. PKC inhibition suppressed the low-dose ANG II effects. High-dose ANG II decreased LMV Na/H exchange activity. PKC inhibition did not affect the high-dose ANG II effects.

The presence of NHE3 protein and activity in LMV was expected, since NHE3 is well-known as the main luminal isoform in proximal tubule cells (5). The present study also shows the presence of NHE2 protein, in agreement with previous observations reporting the presence of NHE2 protein in LMV isolated from rat kidney cortex (9, 13). However, Na/H exchange activity...
in LMV represented NHE3 activity. Indeed, the very small Hoe-694-sensitive transport activity reflects the marginal inhibition of NHE3 activity in agreement with recent data in a similar preparation (29). Indeed, NHE2 protein, mRNA, and transport activity have not been found in the proximal tubule (9, 12, 26). The contamination of the preparation by distal structures is not sufficient to give measurable transport activity, in agreement with previous findings that ~97% of the total luminal surface of tubules in rat renal cortex is brush-border membrane surface (16). The presence in LMV of the two other NHE isoforms described in the kidney, NHE1 and NHE4, may be ruled out, since the latter isoforms are present on the basolateral membranes (6, 8). In addition, the absence of 1 µM Hoe-694-sensitive Na uptake and NHE1 protein in LMV confirmed the absence of contamination of LMV by basolateral membrane vesicles.

PMA acutely increased NHE3 activity, and protein abundance remained unchanged in LMV. The effects of PMA on NHE3 were the net result of increased abundance and activity of PKC isoforms α, δ, and ε. The present study showing an increase of the luminal Na/H exchange activity by PMA is in agreement with the previous results obtained by acutely applying phorbol esters basolaterally or luminally in rat proximal tubule perfused in vivo (19, 25) or by directly applying phorbol esters on LMV isolated from rabbit kidney cortex (27). PMA-induced PKC activation could directly increase the activity of the transporter, since Weinman and his group (28) have shown that PKC-mediated phosphorylation of solubilized luminal membrane proteins elicited a rise in Na/H activity subsequently measured in reconstituted liposomes containing the phosphorylated proteins. However, our results are not in agreement with previous studies using phorbol ester on proximal tubular-like cell lines (20) or in NHE-deficient fibroblast line transfected with NHE3 (15, 18). In the latter studies, phorbol ester acutely decreased NHE3 activity. A possible explanation for this discrepancy may be that the regulation of NHE3 by phorbol esters could be tissue specific, related to different PKC isoform profiles or PKC-specific binding or anchoring proteins in proximal tubules and cultured cells. The loss of a putative cytosolic regulator during the preparation of LMV cannot be excluded but seems less probable with regard to the stimulation of luminal Na/H exchange activity by phorbol esters observed in intact tubules (19, 25).

Low-dose ANG II (10^{-11} M) increased the Na/H exchange activity in LMV, and this effect was suppressed by PKC inhibition, in agreement with our previous studies in cortical tubule suspensions (14). In contrast to the PMA data, ANG II selectively increased PKC-ζ abundance in LMV, whereas the abundance of PKC isoforms α, δ, and ε remained unchanged. The absence of modification of the latter PKC isoforms by low-dose ANG II, i.e., the calcium and diacylglycerol (DAG)-sensitive α-isoform and the DAG-sensitive δ- and ε-isoforms, is compatible with the lack of stimulation of phospholipase C as indicated by the unchanged IP₃ production and cytosolic Ca²⁺ observed in our previous studies with cortical tubule suspensions (22). We have no indication in the present study on the mechanisms of stimulation of the calcium- and DAG-sensitive PKC-ζ by low-dose ANG II. Taken together, the low-dose ANG II and PMA data suggest that NHE3 activity may be stimulated by increase in PKC-ζ alone and by a simultaneous increase in PKC isoforms α, δ, and ε.

In conclusion, PMA acutely increases the NHE3 activity, by translocation of PKC isoforms α, δ, and ε into LMV, whereas low-dose ANG II stimulated NHE3 activity via selective translocation of PKC-ζ. High-dose ANG II inhibited Na/H exchange activity in LMV, and this was not PKC mediated.

Fig. 3. Effect of low-dose ANG II on PKC-ζ abundance in LMV. Cortical tube fragments were treated with or without ANG II (10^{-11} M, 4 min). Cells were homogenized, and LMV were isolated. Ten micrograms of protein from each sample was subjected to 7.5% SDS-PAGE and immunoblotting with specific antibodies (1/1,500 dilution). Molecular mass standard is indicated on left. Results are from 2 representative experiments of 4 total membrane preparations.

Fig. 4. Effect of high-dose ANG II on 22Na uptake. Cortical tubule fragments were treated with ANG II (10^{-7} M) or vehicle, and LMV were subsequently isolated. Outward H⁺ gradient-stimulated 22Na uptake was determined with or without (Tot) 100 µM EIPA. Values, expressed as percent of controls, are means ± SE of 5 membrane preparations; 100% = 4.52 ± 0.59 nmol·mg protein⁻¹·10⁻¹ s⁻¹; EIPA-resistant, 0.59 ± 0.09 and 0.51 ± 0.07 nmol·mg protein⁻¹·10⁻¹ s⁻¹ for controls and ANG II, respectively. Bis, bisindolylmaleimide. **P < 0.01. *P < 0.02. **P < 0.01.
REFERENCES