PMA- and ANG II-induced PKC regulation of the renal Na\(^{+}\)-HCO\(_{3}^{-}\) cotransporter (hkNBCe1)

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Submitted 29 October 2004; accepted in final form 31 August 2005

Perry, Clint, Judith Blaine, Hong Le, and Irina I. Grichtchenko. PMA- and ANG II-induced PKC regulation of the renal Na\(^{+}\)-HCO\(_{3}^{-}\) cotransporter (hkNBCe1). Am J Physiol Renal Physiol 290: F417–F427, 2006. First published September 13, 2005; doi:10.1152/ajprenal.00395.2004.—The renal electrogenic Na\(^{+}\)-HCO\(_{3}^{-}\) cotransporter (hkNBCe1) plays a major role in the bicarbonate reabsorption by the kidney. We examined how PKC and ANG II-activated PKCs regulate hkNBCe1 expression without the ANG II receptors AT1B in Xenopus laevis. We found that 10 nM PMA halved the hkNBCe1 current detected in voltage-clamped oocytes. A PKC-specific inhibitor GF-109203X, and a specific inhibitor of Ca-dependent conventional PKCoβγ, GÖ-6976, significantly reduced PMA inhibition. PMA did not alter surface expression of the cotransporters, but significantly increased hkNBCe1-PKCoβγ membrane association. We found that at 10\(^{-6}\) M, ANG II halved the hkNBCe1 current detected in oocytes coexpressing cotransporters with AT1B. A PKC-specific inhibitor GF-109203X, and a PKCε translocation inhibitor eV1–2 peptide as well as BAPTA-AM (but not GÖ-6976), significantly reduced ANG II inhibition. At 10\(^{-6}\) M, ANG II significantly decreased surface expression of the cotransporters and increased hkNBCe1-PKCe membrane association. Additionally, we found that at 10\(^{-1}\) and 10\(^{-10}\) M ANG II stimulated hkNBCe1 current. This effect was blocked by BAPTA-AM and partially reduced by GF-109203X. We also found that ANG II increased intracellular Ca\(^{2+}\) in fluo 4-loaded oocytes. Our results suggest that 1) PMA inhibition of hkNBCe1 is mediated by Ca-dependent PKCo and 10 nM PMA does not induce downregulation of cotransporter surface expression. 2) ANG II (10\(^{-6}\) M) inhibition of hkNBCe1 is mediated by both Ca-independent PKCe and downregulation of cotransporter surface expression, possibly triggered by intracellular Ca\(^{2+}\) mobilization. 3) Similar to proximal tubule, acute ANG II has a biphasic effect on hkNBCe1 coexpressed with AT1B in X. laevis oocytes.

PKCoβγε; MAPK; intracellular calcium; fluo 4; endocytosis

A major task of the kidneys is to reabsorb HCO\(_{3}^{-}\) to maintain blood at neutral pH. The electrogenic Na\(^{+}\)-HCO\(_{3}^{-}\) cotransporter (NBC1) is responsible for the majority of bicarbonate reabsorption in the proximal tubule (PT) of the kidney. Cloning of the superfamily of sodium-coupled bicarbonate transporters (SCBTs) has revealed distinct putative phosphorylation sites for protein kinases, suggesting that these transporters can be regulated by PKC. There is still little information, however, about the PKC regulation of individual members of the bicarbonate transporter superfamily. The PKC family of serine/threonine kinases has 12 members, divided into three major groups of isoforms. These groups consist of the Ca-dependent conventional isoforms cPKCa, -β1, -βII, and -γ; the Ca-independent novel isoforms nPKCδ, -ε, -η, and -θ and possibly PKCζ, and the atypical isoforms aPKCd1 and -λ, each group of which exhibits somewhat different properties (16).

PMA, a potent PKC activator (4), has been reported to stimulate bicarbonate absorption by acting on the Na\(^{+}\)-HCO\(_{3}^{-}\) transporter in the renal PT (34, 39, 40). PMA has been found to stimulate or inhibit bicarbonate absorption, depending on exposure time (38). Another PKC inducer is the peptide hormone ANG II, which may be derived from the general circulation or synthesized in the kidneys. The Na\(^{+}\)-HCO\(_{3}^{-}\) cotransporter is inhibited by high doses but stimulated by low doses of ANG II in the renal PT (8, 12, 15, 17, 33). It has been reported that PKC is involved in the ANG II-induced regulation of bicarbonate reabsorption in the renal PT (5, 11, 37). PMA and ANG II each induce a distinct pattern of activated PKC isoforms (3, 20, 26), which may have differential roles in the regulation of NBC1. However, the PKC isoforms involved in the PMA- or ANG II-induced regulation of NBC1 are not yet known.

We therefore sought to elucidate the role of PKC and some of its isoforms, as well as the role of intracellular Ca\(^{2+}\), in the PMA- and ANG II-induced regulation of human kidney NBC1 (hkNBCe1) encoded by the SLC4A4 gene expressed in Xenopus laevis oocytes.

EXPERIMENTAL PROCEDURES

Materials. PMA, 4α-phorbol 12,13-didecanoate (4αPDD), GF-109203X, GÖ-6976, the PKCε translocation inhibitor eV1–2 peptide EAVSLKPT, the PKCe translocation inhibitor eV1–2 peptide negative control LSETKPAV, and PD-98059 were purchased from Calbiochem (San Diego, CA). [Asn1,Val5]-ANG II acetate salt was purchased from Sigma (St. Louis, MO). BAPTA-AM, fluo-4, pen-tapotassium salt, and ionicycin were purchased from Molecular Probes (Eugene, OR). Monoclonal anti-PKC (Anti-PKCa, clone M4) was purchased from Upstate USA (Chicago, IL). Monoclonal anti-PKC mouse IgG2a (A-3), polyclonal anti-PKCa (H-300) and anti-PKCε (sc-213) rabbit antibodies, and Protein Plus A/G Agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Living Colors full-length polynolin GFP antibody was purchased from BD Biosciences (San Francisco, CA). Leibovitz’s L-15 Medium and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). EZ-Link Sulfo-NHS-Biotin and immobilized neutravidin biotin binding protein were purchased from Pierce Biotechnology (Rockford, IL). All other chemicals were purchased from Sigma.

Preparation of oocytes. Female X. laevis frogs (NASCO) were anesthetized with 1.5 mg/ml tricaine. The ovarian lobes were surgically removed, dissected, and then treated with 2 mg/ml collagenase type IA in Ca\(^{2+}\)-free ND96-HEPES solution. Oocytes were incubated

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Ang II was made as a 10 mM stock solution in sterile dH2O and diluted with ND96-HEPES solution to the final concentrations before use. As a control, we used 0.1% DMSO solutions in DMSO and diluted with ND96-HEPES to the indicated concentrations. The cDNAs encoding human hkBNC1 and rat AT1B (the kind gift from Dr. L. Pulakat, Bowling Green State University, Bowling Green, OH) were each subcloned into the pGH19 expression vector. A chimera of the EGFP-tagged hkBNC1 cDNA was subcloned into pGH19 (the kind gift from Dr. L. V. Virkki, Institute of Physiology, University of Zurich, Zurich, Switzerland). DNAs were transcribed in vitro using an mMessageMachine kit (Ambion, Austin, TX) to generate synthesized capped mRNAs. Oocytes were injected with 50 nl of 0.5 ng/nl hkBNC1 mRNA; 25 nl of 1 ng/nl hkBNC1 mRNA plus 25 nl 1 ng/nl AT1B mRNA; or 50 nl of dH2O.

Solutions. Nominally bicarbonate-free ND96-HEPES solution contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.50. Bicarbonate-containing solutions were prepared by replacing 33 mM NaCl with 33 mM NaHCO3 and equilibrating with 5% CO2-balanced oxygen. Ca-free ND96-HEPES and bicarbonate-containing solutions were prepared by adding 0.5 mM EGTA. The osmolality of all solutions was ~200 mosmol/kgH2O.

Drug treatments. PMA, 4ePDD, GF-109203X (GF), GO-6976 (GO), PD-98059 (PD), and BAPTA-AM were made as 1,000 × stock solutions in DMDO and diluted with ND96-HEPES solution to the final concentrations before use. As a control, we used 0.1% DMSO. ANG II was made as a 10−3 M stock in sterile dH2O and diluted with ND96-HEPES solution to the final concentrations before use. Where indicated, 8 ng PKCα/V1–2-s peptides, in 24 nl, were each injected into oocytes before the experiments.

Two-electrode oocyte voltage clamp. Oocytes were voltage-clamped at room temperature using a two-electrode oocyte clamp (Warner Instrument, New Haven, CT) and microelectrodes made by pulling borosilicate glass capillary tubing (Warner Instruments) on a microelectrode puller. The cells were impaled with microelectrodes filled with 3 M KCl (resistance = 0.3–1.0 MΩ). The holding potential (Vh) was −50 mV. The currents were filtered at 20 Hz (four-pole Bessel filter) and digitized. An oocyte was placed in a chamber for constant superfusion with a 4-ml/min solution flow. Bath solutions were delivered with syringe pumps (Harvard Apparatus, South Natick, MA), and solutions were switched with pneumatically operated valves (Clippard Instrument Laboratory, Cincinnati, OH).

Biotinylation of surface proteins. Oocytes injected with hkBNC1-EGFP mRNA or dH2O were incubated in the presence or absence of 10 nM PMA for 10 min, and oocytes coinjected with hkBNC1-EGFP and AT1B mRNAs or dH2O were incubated for 20 min in the presence or absence of 10−6 M ANG II. Next, oocytes were incubated in the presence or absence of EZ-Link Sulfo-NHS-Biotin for 1 h at 4°C, and the biotinylated proteins were recovered from the membrane fractions with immobilized neutravidin biotin binding protein by precipitation overnight at 4°C. Proteins were boiled in Laemmli sample buffer and subjected to SDS-PAGE (25) and Western blot analysis using anti-GFP antibodies, or with the beads in the absence of antibody. The beads were washed in high-salt RIPa buffer (RIPA buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.5% Na deoxycholate, 0.1% SDS, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 200 µg/ml PMSF). The membranes were incubated overnight at 4°C with protein AG PLUS-agarose beads and polyclonal rabbit anti-GFP antibodies, or with the beads in the absence of antibody. All averages are reported as means ± SD, along with the number of observations (n). For ratios, averages are presented as log-normal means. The statistical significance of log-normal data was determined using an unpaired Student’s t-test (21). Differences were considered significant at a level of P < 0.05.

RESULTS

Functional characterization of PMA inhibition of hkBNC1 expressed in X. laevis oocytes. To determine the effects of PMA on hkBNC1 function, we injected a synthesized mRNA encoding hkBNC1-EGFP into oocytes and 3 days later performed electrophysiological experiments using a two-electrode oocyte voltage clamp. The EGFP tag did not appear to alter the function of hkBNC1 and action of PMA, because voltage-clamp currents recorded from hkBNC1-EGFP and wild-type hkBNC1 were similar (data not shown). In bicarbonate-buffered solution, each molecule of activated electrogenic hkBNC1 works to pump two HCO3− ions and one Na+ into the cell, thus translocating a net negative charge. This is reflected as a transient outward current in response to the depolarizing step from −50 to 0 mV in voltage-clamped oocytes expressing hkBNC1.

Using oocytes constantly superfused with bicarbonate-buffered solution, a 10-min voltage clamp to −50 mV Vh was applied. When we recorded Ikbc in response to a 60-s depolarization, from −50 to 0 mV, we observed an immediate and large (peak −2 µA) transient outward “control” current (dashed traces marked with arrows in Fig. 1A). After 10-min treatment with 10 nM PMA with or without inhibitors or
BAPTA-AM pretreatment, we recorded a “test” current in response to another depolarization (solid traces in Fig. 1A). We computed a “remaining” (normalized) current by dividing $\Delta I$ (test current after treatment) by $\Delta I_c$ (control current before treatment) (Fig. 1B).

We found that a 10-min treatment with 10 nM PMA caused a significant inhibition of NBC current (typical recording in Fig. 1A, A; bar graph A in Fig. 1B), with the remaining current being $51.0 \pm 8.1\%$ ($n = 9$) of the control current before PMA treatment. We also found that this inhibition was PMA specific, as a 10-min application of 400 nM of the nonactive PMA analog 4oPDD did not inhibit NBC current (Fig. 1A, B; bar graph B in Fig. 1B), with the remaining current being $98.0 \pm 2.5\%$ ($n = 8$) of the controls. In addition, 100 nM GF, a specific inhibitor of PKC, applied together with 10 nM PMA, significantly reduced PMA inhibition of NBC current (Fig. 1A, C; bar graph C in Fig. 1B), with the remaining current being $86.1 \pm 9.0\%$ ($n = 6$) of the controls, indicating that PMA inhibits hkNBC1 in oocytes via a PKC signaling pathway.

To identify the PKC isoforms involved in PMA inhibition of hkNBC1, we preincubated the cells for 25 min with 200 nM GO, a specific inhibitor of Ca-dependent conventional PKC$\alpha\beta\gamma$, which significantly reduced PMA inhibition of NBC current (Fig. 1A, D; bar graph D in Fig. 1B), with the remaining current being $81.0 \pm 7.2\%$ ($n = 6$) of the controls. These findings clearly demonstrate that PMA-induced Ca-dependent conventional PKC$\alpha\beta\gamma$ isoforms are involved in hkNBC1 inhibition in X. laevis oocytes.

To examine the role of Ca$^{2+}$ in PMA inhibition of NBC current, we chelated intracellular Ca$^{2+}$ by incubating oocytes for 30 min in 50 $\mu$M cell-permeable BAPTA-AM. This chelation reduced PMA inhibition of NBC current (Fig. 1A, E; bar graph E in Fig. 1B), with the remaining current being $74.1 \pm 5.4\%$ ($n = 9$) of the controls. Next, we applied 100 nM GF together with 10 nM PMA to the BAPTA-AM-pretreated oocytes. Ca$^{2+}$ chelation did not alter GF-induced reduction of PMA inhibition of NBC (Fig. 1A, F; bar graph F in Fig. 1B), with the remaining current being $83.0 \pm 12.2\%$ ($n = 6$) of the controls, indicating that there is no summation of GF and BAPTA effects. Similarly, a separate set of experiments indicated that there is no summation of GO and BAPTA effects in the presence of PMA, with the remaining current being $74.6 \pm 4.1\%$ ($n = 5$) of the controls (Fig. 1A, G; bar graph G in Fig. 1B). We also monitored intracellular Ca$^{2+}$ in flou 4-injected oocytes expressing hkNBC1; 10 nM PMA does not increase fluorescence intensity of flou 4-loaded oocytes (see Fig. 7A), indicating stable cytosolic Ca$^{2+}$ concentration during PMA application. These findings clearly demonstrate that Ca$^{2+}$ signaling is not involved in PMA inhibition of hkNBC1 expressed in X. laevis oocytes.

Functional characterization of ANG II inhibition of hkNBC1 coexpressed with AT1B in X. laevis oocytes. To determine the effects of high concentration ANG II ($10^{-6}$ M) on NBC current, we coinjected oocytes with mRNAs encoding hkNBC1-EGFP and rat AT1B. Again, the EGFP tag did not appear to alter the function of hkNBC1 and the action of ANG II, because voltage-clamp currents recorded from hkNBC1-EGFP or wild-type hkNBC1 coexpressed with AT1B were similar (data not shown). Using the method described above, we initially tested oocytes expressing only hkNBC1 and recorded the “control” current (dashed line, Fig. 2A, A). After treatment for 20 min with $10^{-6}$ M ANG II, we recorded the
“test” current in response to another depolarizing step (solid trace, Fig. 2A, A). We found that, at this concentration, ANG II had no effect on hkNBCe1 current [remaining NBC current, 97.5 ± 10.2% (n = 6) of the control; bar graph A in Fig. 2B]. When we tested oocytes coexpressing hkNBCe1 with AT1B, we found that a 20-min treatment with 10^{-6} M ANG II caused a significant inhibition of NBC current with the remaining current being 50.6 ± 7.7% (n = 11) of the control current before ANG II treatment (Fig. 2A, B; bar graph B in Fig. 2B). We found that 100 nM G04 applied with 10^{-6} M ANG II blocks ANG II-induced inhibition, with the remaining current being 99.5 ± 9.0% (n = 6) of the control (Fig. 2A, C; bar graph C in Fig. 2B), indicating that high-concentration ANG II inhibits NBC current via the PKC signaling pathway. We incubated oocytes with 200 nM G04 for 25 min before and during a 20-min treatment with ANG II. G04 had no effect on ANG II inhibition of NBC current (Fig. 2A, D). Surprisingly, G04 slightly enhanced the inhibitory effect of 10^{-6} M ANG II, with the remaining NBC current being 42.9 ± 3.0% (n = 6) of the control (bar graph D in Fig. 2B). These results indicate that Ca-dependent PKC isoforms are not involved in ANG II inhibition of NBC current.

High doses of ANG II have been reported to activate the Ca-independent novel PKCζ isoform in rat renal PT (20). To determine whether PKCζ is involved in ANG II-induced inhibition, we injected oocytes with 8 ng of the PKCζ translocation inhibitor eV1–2 peptide EAVSLKPT or its negative control eV1–2–s LSETKPAV. In experiments similar to the one shown in Fig. 2A, E and Fig. 2A, F, we found that EAVSLKPT significantly reduced ANG II inhibition of NBC current to 92.7 ± 8.4% (n = 6) of control (P < 0.005; bar graph E in Fig. 2B). In contrast, LSETKPAV had only a slight effect, with the remaining NBC current being 67.6 ± 3.0% (n = 6) of control (bar graph F in Fig. 2B). These findings suggest that PKCζ is involved in ANG II inhibition of NBC current.

We monitored intracellular Ca^{2+} in fluo 4-loaded oocytes coexpressing hkNBCe1 and AT1B. We detected a large transient increase (peaking at a 3.8-fold increase of normalized fluorescence within first 1–3 min) followed by a sustained elevation of intracellular Ca^{2+} (~1.8-fold of normalized fluorescence, within last 10–25 min) in oocytes treated with 10^{-6} M ANG II, but not in oocytes preincubated for 30 min with 50 μM BAPTA-AM (see Fig. 7, B, C, and D). Next, voltage-clamped oocytes were incubated for 30 min with 50 μM BAPTA-AM, which significantly reduced inhibition of NBC current induced by 10^{-6} M ANG II (Fig. 2A, G) to 88.1 ± 6.2% (n = 8) of control (bar graph G in Fig. 2B). These results suggest that intracellular Ca^{2+} is involved in high-concentration ANG II inhibition of NBC current.

AT1 stimulation has been reported to activate the MAPK cascades (35). To determine whether MAPK is involved in ANG II inhibition, we pretreated oocytes for 50 min with 50 μM PD, a selective and cell-permeable MAPK (MEK) inhibitor, followed by a 20-min treatment with 10^{-6} M ANG II plus 50 μM PD (Fig. 2A, H). We found that ANG II inhibition of NBC current was significantly reduced, with the remaining NBC current being 80.5 ± 10.2% (n = 6) of control (bar graph H in Fig. 2B). This finding is in agreement with earlier reports.
that MAPK is involved in the ANG II regulation of endogenous Na\(^+\)-HCO\(_3\)^\(-\) cotransporter in the heart and kidney (1, 31).

Quantitation of plasma membrane hkNCBe1 proteins in the presence of PMA. To investigate whether PMA-activated PKC inhibits NBC current by inducing removal of hkNCBe1-EGFP proteins from the plasma membrane, we performed surface biotinylation using a membrane-impermeable derivative of biotin (sulfo-NHS-biotin). The EGFP tag did not appear to alter the level of the hkNCBe1 biotinylated protein (data not shown). Western blot analysis with anti-GFP monoclonal antibodies detected biotinylated hkNCBe1-EGFP protein as a ~150-kDa band on SDS-PAGE (Fig. 3A); the difference in size between this band and the 130-kDa glycosylated hkNCBe1 (7) corresponds to the ~26-kDa EGFP. We found that the intensity of the hkNCBe1 band from PMA-treated oocytes (Fig. 3A, lane 4) was 103.4 ± 13.2% (n = 6) of that of untreated cells (Fig. 3A, lane 3), indicating that a 10-min treatment with 10 nM PMA does not induce removal of the hkNCBe1 from the membrane. Negative controls (Fig. 3A, lanes 1, 2, 5, and 6) showed a complete absence of biotinylated hkNCBe1.

To further show that short treatment with 10 nM PMA does not induce downregulation of the surface expression level of hkNCBe1 cotransporters, oocytes expressing hkNCBe1-EGFP were imaged near membrane faces using confocal laser-scanning microscopy (Fig. 4A). The mean fluorescence signal was obtained from the XY section within the region of interest (ROI) (dotted in Fig. 4A). The mean value for nontreated oocytes (Fig. 4Aa) was used to normalize the fluorescence signal in oocytes treated for 10 min with 10 nM PMA (Fig. 4Ab). We found that the hkNCBe1-EGFP fluorescence in both sets of cells was the same, with the near-membrane fluorescence in PMA-treated oocytes being 97.1 ± 15.4% (n = 3) of that of untreated cells.

Quantitation of plasma membrane hkNCBe1 in the presence of ANG II. To determine whether ANG II decreases surface expression of hkNCBe1 cotransporters, we performed similar cell surface-biotinylation assays (e.g., Fig. 3B). By Western blot analysis, we found that the intensity of the biotinylated hkNCBe1-EGFP band from ANG II-treated oocytes (Fig. 3B, lane 4) was significantly decreased to 68.6 ± 10.0% (n = 4) of that observed in untreated cells (Fig. 3B, lane 3), indicating that a 20-min treatment with 10\(^{-6}\) M ANG II inhibited hkNCBe1 by partial removal of the cotransporter population from the membrane. Negative controls (Fig. 3B, lanes 1, 2, 5, and 6) showed a complete absence of biotinylated hkNCBe1.

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To further show that $10^{-6}$ M ANG II downregulates surface expression level of hkNBCe1 cotransporters, we performed confocal microscopy experiments, as described above (Fig. 4B). We found that the hkNBCe1-EGFP fluorescence near the plasma membrane of oocytes treated for 20 min with $10^{-6}$ M ANG II (Fig. 4Bb) was significantly lower (65.3 ± 18.7%, $n = 4$) than that of untreated oocytes (Fig. 4Ba).

Coprecipitation of hkNBCe1 and PKC from oocyte membrane fractions. We performed communoprecipitation to determine whether there was an association between hkNBCe1 proteins and endogenous PKC proteins at the plasma membrane of the X. laevis oocytes. One group of oocytes expressing hkNBCe1-EGFP or injected with dH2O was treated with 10 nM PMA for 10 min. Another group of cells coexpressing hkNBCe1-EGFP and AT1B or dH2O-injected was treated with 10 nM PMA (b). The fluorescence of hkNBCe1-EGFP near the plasma membrane at a scaling factor of 0.23 μM, with 488-nm excitation and 510-nm emission, was acquired with an LSM510 microscope (Zeiss) using a plan-Neofluor, water-immersion objective ×25/0.8 mm with argon laser for excitation. In PMA-treated cells, the fluorescence intensity in the region of interest (ROI, inside the white dotted area) was 97.1 ± 15.4% ($n = 3$) of that in untreated cells. B: ANG II at $10^{-6}$ M reduces the fluorescence of hkNBCe1-EGFP near the plasma membrane. Confocal microscopy images of oocytes coexpressing hkNBCe1-EGFP and AT1B show a significant reduction in hkNBCe1-EGFP fluorescence after treatment for 20 min with $10^{-6}$ M ANG II (b) compared with untreated oocytes (a). The fluorescence of hkNBCe1-EGFP near the plasma membrane at a scaling factor of 0.53 μM was acquired as above. In ANG II-treated cells, the fluorescence intensity in the ROI was 65.3 ± 18.7% ($n = 4$) of that in untreated cells.

PMA treatment increased the amount of PKCαβγ precipitated. The intensity of the PKCαβγ band from PMA-treated oocytes (Fig. 5A, lane 1) was 302.6 ± 53.3% ($n = 4$) of that from untreated oocytes (Fig. 5, lane 2). We also found that 10^{-6} M ANG II increased the amount of PKCαβγ precipitated. The intensity of the PKCαβγ band from ANG II-treated oocytes (Fig. 5B, lanes 1 and 2) was 270.4 ± 59.0% ($n = 4$) of that from untreated oocytes (Fig. 5B, lane 1).

Biphasic effect of ANG II. When we tested oocytes expressing hkNBCe1 without AT1B, we found that neither $10^{-11}$ nor $10^{-6}$ M ANG II altered NBC current, with the remaining current being 92.2 ± 9% ($n = 6$) and 97.5 ± 18.2% ($n = 6$), respectively, of the control (data not shown). When we tested oocytes coexpressing hkNBCe1 with AT1B, we found that a 20-min treatment with $10^{-11}$ or $10^{-10}$ M ANG II caused a moderate stimulation of NBC current, with the remaining current being 126.2 ± 6.6% ($n = 6$) and 121.6 ± 10.2% ($n = 6$), respectively, of the control (Fig. 6, bar graphs C1 and D1). In contrast, $10^{-9}$ M ANG II had no effect on hkNBCe1 [remaining current, 97.3 ± 6.0% ($n = 6$) of the control; Fig. 6, bar graph E1], whereas 20-min treatments with $10^{-8}$ and $10^{-6}$ M ANG II significantly inhibited hkNBCe1, with the remaining...
currents being 62.8 ± 9.6 (n = 6) and 50.6 ± 7.7% (n = 11), respectively, of the control (Fig. 6, bar graphs F1 and G1). These results indicate that ANG II has a biphasic effect on hkNBCe1 coexpressed with AT1B in oocytes.

We found that stimulation by 10^{-11} M ANG II was slightly inhibited by 100 nM GF but was completely blocked by 50 μM BAPTA-AM, with the remaining current being 119.2 ± 9.6 (n = 6) and 101.7 ± 8.4% (n = 6), respectively, of control (bar graphs C2 and C3). The smaller stimulatory effect of 10^{-10} M ANG II (Fig. 6, bar graph D1) was significantly inhibited by GF [remaining current, 107.5 ± 9.4% (n = 6) of control; bar graph D2] and blocked by BAPTA [remaining current, 98.2 ± 8.8% (n = 6) of control; bar graph D3]. Neither GF nor BAPTA applied with 10^{-9} M ANG II had an effect on hkNBCe1, with the remaining current being 104.2 ± 4.8 (n = 6) and 87.1 ± 6.6% (n = 6), respectively, of control (bar graphs E2 and E3). We found that inhibition by 10^{-8} M ANG II was significantly reduced by GF [remaining current: 88.3 ± 10.8% (n = 6) of control; bar graph F2] and by BAPTA [remaining current: 87.8 ± 8.8% (n = 6) of control; bar graph F3]. Results observed at 10^{-7} M ANG II were already discussed above (see Fig. 2B, bar graphs B, C, G with the same

Fig. 5. A: PMA increases the intensity of the PKCaβγ band coimmunoprecipitated with hkNBCe1 from oocyte membrane fractions. Oocytes injected with hkNBCe1 or dH2O and incubated for 10 min in the presence or absence of 10 nM PMA were homogenized, and each membrane fraction was immunoprecipitated with GFP polyclonal antibodies, followed by Western immunoblot analysis using monoclonal anti-GFP (1:1,000; top) and anti-PKCαβγ antibodies (specific to PKCaβγ) (1:1,000; bottom). Lane 1: oocytes expressing hkNBCe1-EGFP and treated with PMA. Lane 2: oocytes expressing hkNBCe1-EGFP not treated with PMA. Lane 3: control dH2O-injected oocytes treated with PMA. Lane 4: control H2O-injected oocytes not treated with PMA. Note the increase in the intensity of the PKCaβγ band in the PMA-treated (302.6 ± 53.3%, n = 4) compared with untreated oocytes. B: ANG II increases the intensity of the PKCe band coimmunoprecipitated with hkNBCe1 from oocyte membrane fractions. Oocytes coinfected with hkNBCe1 and AT1B and incubated for 20 min in the presence or absence of 10^{-6} M ANG II, followed by Western analysis using monoclonal anti-GFP (1:1,000; top) and anti-PKCe antibodies (1:1,000; bottom). Lane 1: oocytes coexpressing hkNBCe1-EGFP with AT1B not treated with ANG II. Lane 2: oocytes coexpressing hkNBCe1-EGFP with AT1B and treated with ANG II. Lane 3: control H2O-injected oocytes treated with ANG II. Lane 4: control H2O-injected oocytes not treated with ANG II. Note the increase in the intensity of the PKCe band in the ANG II-treated (270.4 ± 59.0%, n = 4) compared with untreated oocytes.

Fig. 6. Biphasic effect of ANG II on hkNBCe1 coexpressed with AT1B in oocytes: role of PKC and intracellular Ca^{2+}. Bars representing means ± SD of relative NBC peak currents acquired as the ratio of test to control current for 6 (11 in bar G1) experiments obtained from oocytes coexpressing hkNBCe1 and AT1B and treated with ANG II at concentrations of 10^{-11} M (C1), 10^{-10} M (D1), 10^{-9} M (E1), 10^{-8} M (F1), and 10^{-7} M (G1). Bars C2, D2, E2, F2, and G2: 100 nM GF was applied together with the indicated concentration of ANG II for 20 min. Bars C3, D3, E3, F3, and G3: intracellular Ca^{2+} was chelated by a 30-min pretreatment with 50 μM BAPTA-AM, followed by treatment with the indicated concentration of ANG II for 20 min. *P < 0.005. **P < 0.05 by Student’s t-test.
Fig. 7. Ca\(^{2+}\) signaling in fluo 4-loaded oocytes. A: PMA does not increase cytosolic Ca\(^{2+}\) in oocytes expressing hkNBCe1. Serial confocal microscope images show similar fluorescence of Ca-sensitive fluo 4 dye in the same oocyte before and 40, 300, 600, and 900 s after 10 nM PMA. The fluorescence intensity in the ROI (inside the white dotted area) after PMA was 97.4 ± 2.4% (n = 4) of that before. B: ANG II increases cytosolic Ca\(^{2+}\) in oocytes coexpressing hkNBCe1 with AT\(_{1B}\). Serial confocal images show significant increase in fluorescence 40, 80, 180, 240, and 300 s after 10\(^{-6}\) M ANG II. The fluorescence intensity in the ROI 80–120 s after ANG II was 373.5 ± 4.23% (n = 3) of that before. The fluorescence intensity in the ROI 12–20 min (images not shown) after ANG II was 179.0 ± 1.5% (n = 3) of that before. C: 50 μM BAPTA-AM blocks ANG II increase in cytosolic Ca\(^{2+}\) in oocytes coexpressing hkNBCe1 with AT\(_{1B}\). Serial confocal images show similar fluorescence before BAPTA, 30 min after BAPTA and before ANG II, and 40, 80, 240, and 300 s after ANG II. In images taken of Ca-chelated oocytes after treatment with 10\(^{-6}\) M ANG II, the fluorescence intensity in the ROI was 97.0 ± 2.7% (n = 3) of that before. A, B, and C: Oocyte diameter within shown focal plane is 465–580 μm, and depth of focus is 8–10 μm. D: Changes in normalized fluorescence (F/F\(_{0}\)) of Ca-sensitive fluo 4 taken every 20 s. • 10 nM PMA (shown in A); ○ 10\(^{-6}\) M ANG II (shown in B); □ BAPTA with 10\(^{-6}\) M ANG II (shown in C). We also measured cytosolic Ca\(^{2+}\) in 10\(^{-10}\) M ANG II-treated oocytes coexpressing hkNBCe1 and AT\(_{1B}\) (images are not shown); ▲ 10\(^{-10}\) M ANG II; and △ BAPTA with 10\(^{-10}\) M ANG II.
data shown in Fig. 6, bar graphs G1–3). Our results suggest that both PKC pathway and Ca\(^{2+}\) signaling seem to play important roles in the biphasic effect of ANG II on hkNBCe1 coexpressed with AT\(_{1B}\) in X. laevis oocytes.

**DISCUSSION**

**PKC inhibition of hkNBCe1.** At low concentrations, PMA is a potent PKC activator in many cells, including X. laevis oocytes (4, 41). We showed here that for treatment of 10 min with 10 nM PMA results in a ~50% reduction in the activity of the renal electrogenic Na\(^{+}\)-HCO\(_{3}^{-}\) cotransporter, as assayed by the amplitudes of the current via hkNBCe1 in response to a depolarizing step in voltage-clamped oocytes. This PMA-specific inhibition is mediated by PKC, as indicated by the observation that GÖ significantly reduced hkNBCe1 inhibition. Moreover, PKC inhibition is mediated by Ca-dependent conventional PKCaβγ isofoms, as indicated by the observation that GÖ significantly reduced hkNBCe1 inhibition. PKC has been reported to cause a rapid redistribution of PKC activity from the cytosol to the membrane fraction of cells (22). Because hkNBCe1 localizes to membranes, the translocation of PKC makes it more accessible for interaction with the cotransporter. In PMA-untreated oocytes, we detected endogenous isoforms of PKCaβγ in the hkNBCe1-EGFP immunoprecipitates from the membrane fractions, suggesting a basal level of membrane association between hkNBCe1 and PKCaβγ. A 10-min treatment with 10 nM PMA enhanced the hkNBCe1-PKCaβγ interaction threefold. PMA has been reported to have differential effects on the surface expression of membrane proteins. For example, PMA induces retrieval of renal Na\(^{+}\)/dicarboxylate cotransporters (29), type II Na\(^{+}\)-phosphate cotransporters (14), and GABA\(_{C}\) receptors (24) expressed in X. laevis oocytes. In contrast, PMA does not alter the level of surface expression of CFTR expressed in X. laevis oocytes (6). Therefore, we performed surface biotinylation and confocal fluorescent imaging experiments to determine whether PMA-activated PKC alters the membrane expression of the hkNBCe1 cotransporters. Our data clearly indicate that a 10-min treatment with 10 nM PMA does not cause retrieval of the cotransporter from the membrane of X. laevis oocytes. Thus taken together, our electrophysiological, coprecipitation, biotinylation, and imaging data strongly suggest that PMA-activated PKCaβγ isoforms may inhibit hkNBCe1 via protein-protein interaction.

Importantly, we eliminated the possibility that Ca\(^{2+}\) signaling was involved in the observed PMA inhibition of NBC current. First, we directly monitored intracellular Ca\(^{2+}\) in PMA-treated fluo 4-loaded oocytes and detected no cytosolic Ca\(^{2+}\) elevation. Consequently, our observations that BAPTA reduces PMA inhibition of NBC current may be due to the reported ability of BAPTA to inactivate PKC (9). Second, we observed that in BAPTA-treated oocytes, both PKC inhibitors (GF or GÖ) reduced PMA inhibition of NBC current similarly to that in BAPTA-untreated oocytes, suggesting that Ca\(^{2+}\) signaling is not involved in PMA inhibition. How can Ca-dependent PKC isoforms be activated in the absence of cytosolic Ca\(^{2+}\) increase? It has been suggested that PMA directly binds to the diacylglycerol binding domain of PKC and activates Ca-dependent PKC isoforms in the absence of concurrent Ca\(^{2+}\) occupation of the Ca-binding domain (28).

To summarize, our results suggest that 1) Ca-dependent PKCaβγ isoforms mediate PMA-induced inhibition of hkNBCe1, 2) PMA increases the membrane interaction of PKCaβγ with hkNBCe1, and 3) PMA-activated PKC does not induce downregulation of hkNBCe1 cotransporter surface expression level (see model in Fig. 8).

**ANG II inhibition of hkNBCe1.** In the renal PT, ANG II binds to the AT\(_{1}\) and interacts primarily with G\(_{i}\) proteins, leading to activation of PKC and to mobilization of Ca\(^{2+}\) from intracellular stores (13). X. laevis oocytes lack endogenous receptors for ANG II but have powerful G\(_{i}\) protein-linked PKC and Ca\(^{2+}\) signaling pathways that can be activated by agonists of recombinant mammalian receptors (2, 10, 18, 23, 36). Therefore, we heterologously coexpressed the human hkNBCe1 with the rat AT\(_{1B}\) receptor in X. laevis oocytes. We found that, at 10\(^{-6}\) M, ANG II acting via AT\(_{1B}\) significantly inhibited NBC current. Our results indicate that this occurs via the PKC signaling pathway, as GF blocked ANG II inhibition of NBC current. To identify PKC isoforms involved, we used several isoform-specific inhibitors. We found that GÖ failed to prevent the premature Ca\(^{2+}\) influx that is responsible for downregulating NBC current. Thus PMA does not increase cytosolic Ca\(^{2+}\) and does not trigger downregulation of the surface expression level of cotransporters. ANG II binds to the AT\(_{1}\) receptor, activating PKC and Ca\(^{2+}\) signaling pathways. ANG II-activated Ca-independent novel PKCe isoform inhibits hkNBCe1 via a protein-protein interaction resulting in possible phosphorylation of the cotransporter. ANG II increases cytosolic Ca\(^{2+}\) may downregulate hkNBCe1 by triggering its internalization from the cell surface or inhibiting its recycling back to the cell surface.

Fig. 8. Putative model of PMA and ANG II inhibition of hkNBCe1. PMA diffuses across the cell membrane and directly activates Ca-dependent PKCaβγ isoforms. PKCaβγ isoforms inhibit hkNBCe1 via a protein-protein interaction resulting in possible phosphorylation of the cotransporter. PMA does not increase cytosolic Ca\(^{2+}\) and does not trigger downregulation of the surface expression level of cotransporters. ANG II binds to the AT\(_{1}\) receptor, activating PKC and Ca\(^{2+}\) signaling pathways. ANG II-activated Ca-independent novel PKCe isoform inhibits hkNBCe1 via a protein-protein interaction resulting in possible phosphorylation of the cotransporter. ANG II-increased cytosolic Ca\(^{2+}\) may downregulate hkNBCe1 by triggering its internalization from the cell surface or inhibiting its recycling back to the cell surface.
ANG II inhibition. Thus, unlike PMA inhibition, ANG II inhibition of hNBCe1 is not mediated by Ca-dependent PKCaβγ. Utilizing the highly specific PKCe inhibitor eV1–2, an octapeptide (EAVSLKPT) derived from the receptor for activated C kinase (Rack)-binding site of PKCe (19), we observed almost complete prevention of ANG II inhibition. At the same time the inactive, scrambled analog, eV1–2-s (LSETKPAV), had a much smaller effect. Our results strongly suggest that the PKC pathway acting via the PKCe isofrom is involved in ANG II inhibition of hNBCe1 coexpressed with AT1B in X. laevis oocytes. Furthermore, we detected an endogenous PKCe band in the hNBCe1-EGFP immunoprecipitates from membrane fractions, suggesting a basal level of membrane association between hNBCe1 and PKCe. A 20-min treatment with 10^{-6} M ANG II enhanced the hNBCe1-PKCe interaction 2.7-fold.

Using a cell surface-biotinylation assay followed by Western blot analysis, we observed that treatment with 10^{-6} M ANG II for 20 min significantly decreased the intensity of the biotinylated hNBCe1-EGFP band. These findings were confirmed by confocal laser-scanning microscopy in living cells using biotinylated hNBCe1-EGFP band. These findings were confirmed by confocal laser-scanning microscopy in living cells using biotinylated hNBCe1-EGFP band.

To summarize, our results suggest that 1) Ca-insensitive PKCe isofrom mediates ANG II-induced inhibition of hNBCe1, 2) ANG II increases the membrane interaction of PKCe with hNBCe1, and 3) ANG II-induced intracellular Ca^{2+} elevation may trigger downregulation of the surface expression level of cotransporters in oocytes coexpressing hNBCe1 and AT1B (see model in Fig. 8).

Here, we present data that demonstrate that ANG II regulation of NBC1 in oocytes is similar to that in native epithelia. Specifically, ANG II has a biphasic effect on NBC1 in both oocytes (Fig. 6) and renal PT (8, 12, 15, 17, 33). In the mammalian kidney, however, the ANG II regulation of NBC1 is complicated by the expression of two types of AT receptors and several variants of electrogenic Na^{+}-HCO_{3}^{-} cotransporters (30, 32). Nevertheless, presented data are valuable for the identification of key molecular mechanisms responsible for hormonal regulation of bicarbonate absorption in mammalian kidneys.

ACKNOWLEDGMENTS

We thank Dr. L. Pulakat (Bowling Green State University, Bowling Green, OH) for providing the AT1B cDNA construct and Drs. A. Newton, S. R. Levinson, N. R. Zahniser, and A. Zweifach for helpful advice. We also thank R. Khera, C. Patel, D. Siino, and M. E. Kronberg for technical support.

GRANTS

This work was supported by a Howard Hughes Medical Institute Grant (HHMI 76200-550102) to Dr. I. I. Grichtchenko.

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