ANG II and calmodulin/CaMKII regulate surface expression and functional activity of NBCe1 via separate means

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Perry C, Le H, Grichtchenko II. ANG II and calmodulin/CaMKII regulate surface expression and functional activity of NBCe1 via separate means. Am J Physiol Renal Physiol 293: F68–F77, 2007. First published March 20, 2007; doi:10.1152/ajprenal.00454.2006.—We recently reported that ANG II inhibits NBCe1 current and surface expression in Xenopus laevis oocytes (Perry C, Blaine J, Le H, and Grichtchenko II. Am J Physiol Renal Physiol 290: F417–F427, 2006). Here, we investigated mechanisms of ANG II-induced changes in NBCe1 surface expression. We showed that the PKC inhibitor GF109203X blocks and EGTA reduces surface cotransporter loss in ANG II-treated oocytes, suggesting roles for PKC and Ca2+. Using the endosomal marker FM 4-64 and enhanced green fluorescent protein (EGFP)-tagged NBCe1, we showed that ANG II stimulates endocytosis of NBCe1. To eliminate the possibility that ANG II inhibits NBCe1 recycling, we demonstrated that the recycling inhibitor monensin decreases surface expression, accumulates NBCe1-EGFP in endosomes, and inhibits NBCe1 current. Monensin and ANG II applied together produce greater inhibition of NBCe1 current than either did alone. This additive effect of monensin and ANG II suggests that ANG II stimulates internalization of NBCe1. We used the calmodulin (CaM) antagonist W13, which controls recycling by blocking the exit of the endocytosed cargo from early endosomes, to determine the role of CaM in NBCe1 trafficking. We demonstrated that W13 decreases surface expression of NBCe1, accumulates NBCe1-EGFP in endosomal-like formations, and inhibits NBCe1 current. W13 and ANG II applied together produce greater inhibition of NBCe1 current than either does alone, while W13 and monensin applied together do not. The additive effect of ANG II and W13 and lack of additive effect of monensin and W13 suggest that CaM is not involved in ANG II stimulation of internalization but controls recycling of endocytosed NBCe1. The CaM-activated enzyme CaM kinase II (CaMKII) applied with ANG II also gives an additive inhibitory effect, suggesting a role for CaMKII in NBCe1 recycling.

W13; monensin; FM 4-64; trafficking; endocytosis

NBCe1 is abundant in many tissues (30). This membrane protein plays a major role in the regulation of intracellular pH and in absorption and secretion of HCO3− (6, 29, 34). The amount of NBCe1 at the cell surface is a critical determinant of cotransporter function. Previously, we showed that ANG II inhibits NBCe1 current (I NBC) and decreases the level of surface expression of NBCe1 in Xenopus laevis oocytes (28). Endocytosis is a common phenomenon that controls the level of surface expression of many membrane proteins. X. laevis oocytes have been successfully used to study endocytosis of many membrane proteins, including the thiazide-sensitive Na–Cl cotransporter (NCCT), ROMK channels, EAAT1 transporters, GABA(A) receptors, the epithelial Na+ channels (ENaCs), and the CFTR Cl channels (5, 7, 8, 12, 20, 33, 41, 42). However, before our study nothing was known of the trafficking changes induced by ANG II which result in inhibition of NBCe1. Here, we utilized the properties of FM 4-64 and monensin to test our hypothesis that ANG II induces endocytosis of NBCe1 in X. laevis oocytes. The impermeant, lipophilic styryl dye FM 4-64 partitions into the plasma membrane and is a well-established fluorescent endocytotic marker (4, 19). The monovalent carboxylic ionophore monensin raises pH within endosomes and inhibits recycling of internalized proteins to the plasma membrane (3, 37, 40).

We reported earlier that ANG II inhibits I NBC in a PKC- and Ca2+-dependent manner (28). One objective of the current study was to use the PKC inhibitor GF109203X and Ca2+ chelator EGTA to determine the roles of PKC and Ca in ANG II-induced loss of NBCe1 from the cell surface. It has been shown that the calcium-binding protein calmodulin (CaM) and CaM kinase II (CaMKII) are involved in the regulation of functional activity of renal and intestinal NBCe1 (2, 10, 31, 32). It is also known that CaM controls the recycling step of the endocytic pathway of several membrane proteins (9, 14). Furthermore, its antagonist, W13, blocks the exit of internalized cargo from early endosomes, resulting in the formation of large endosomes (1, 23, 39). However, before our study nothing was known of the involvement of CaM or CaMKII in the trafficking of NBCe1. Here, we utilized inhibitors W13 and KN93 to determine the roles of CaM and CaMKII in the trafficking of NBCe1.

Through biotinylation, confocal fluorescent microscopy in live cells, and two-electrode voltage clamp, we found that ANG II and CaM/CaMKII regulate endocytosis of NBCe1 via separate mechanisms: ANG II induces internalization in a PKC- and Ca2+-dependent manner, while CaM/CaMKII controls recycling of NBCe1.

EXPERIMENTAL PROCEDURES

Materials. GF109203X, W-13, KN93, monensin, and [Asn1,Val5]ANG II acetate salt were purchased from Sigma (St. Louis, MO). Living Colors full-length monoclonal green fluorescent protein (GFP) antibody was purchased from BD Biosciences/Clontech (Palo Alto, CA). Leibovitz’s L-15 medium and penicillin-streptomycin and the styryl dye FM4-64, 5-((and)-6-carboxy-2',7'-dichlorofluorescein diacetate (CDDCDA) 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. All experimental procedures

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The cDNAs encoding human hkBNC1 and rat AT1B (the kind gift of Dr. Lakshmi R. Pulakat, Bowling Green State University, Bowling Green, OH) were each subcloned into the pGHL9 expression vector (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/ml AT1B mRNA plus 25 nl of 1 ng/ml AT1B mRNA, or 50 nl of dH2O.

Drug treatments. W13 was made as a 1,000 × stock in sterile dH2O, KN93 was made as a 1,000 × stock solutions in DMSO, and monensin was made as a 10,000 × stock solution in methanol. All three were diluted with ND96-HEPES solution to the final concentrations before use. As a control, we used 0.1% DMSO and 0.01% methanol. ANG II was made as a 1,000 × stock in sterile dH2O and diluted with ND96-HEPES solution to the final concentrations before use. Where indicated, 50 μM monensin was applied overnight to oocytes kept at 18°C. Where indicated, 50 nl of 50 mM EGTA were injected into oocytes before the experiments.

Two-electrode oocyte voltage clamp. As described previously (28), we injected synthesized mRNAs encoding NBCe1-EGFP and AT1 into oocytes and 3 days later performed electrophysiological experiments. 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). We clamped oocytes to a −50 mV holding potential and measured INaCl in response to a 60-s depolarization, from −50 mV to 0 mV. The currents were filtered at 20 Hz (4-pole Bessel filter) and digitized. An oocyte was placed in a chamber with a 4 ml/min constant superfusion. 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. As described previously (28), oocytes injected with hkBNC1-EGFP mRNA, or dH2O, or coinjected with hkBNC1-EGFP and AT1B mRNAs and after 3 days were incubated for 20 min with 1 μM ANG II alone, 1 μM ANG II with 100 nM GF, or 1 μM ANG II was added to oocytes injected with 50 nl of 50 mM EGTA. In separate experiments oocytes were incubated for 50 min with 100 μM W13 or 50 μM KN93. In separate experiments, oocytes were treated overnight with 50 μM monensin. 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 (21). The hkBNC1-EGFP bands were detected by Western blot analysis with monoclonal anti-GFP antibodies using Kodak Image Station 440CF. The intensity of the hkBNC1 bands was measured using ImageJ software (http://rsb.info.nih.gov/ij/). As a control, we used biotinylated oocytes for immunoprecipitation without avidin and non-biotinylated oocytes immunoprecipitated with avidin.

Confocal microscopy in live oocytes. Fluorescent images of “green” NBCe1-EGFP and “red” styryl dye FM 4-64 were captured in a confocal XY section of the plasma membrane by excitation at 488 nm and emission at 510 nm (GF) and by excitation at 546 nm and emission at 740 nm (FM 4-64). Brightfields were taken in the same confocal XY sections of the plasma membrane in series with fluorescent images. For brightfields, we used an argon laser to illuminate the oocyte and captured the image using a channel without an emission filter. We used a LSM510 microscope (Carl Zeiss Laser Scanning System, available at Light Microscopy Facility at University of Colorado and Denver Health Sciences Center; see http://www.uchsc.edu/lms/lightmicroscopy/) with a ×25 water-immersion objective (NA 0.8 mm) and argon (“green” EGFP) and HeNe1 (‘red” FM 4-64) lasers for excitation. Fluorescence was acquired with Carl Zeiss LSM Image software.

Data acquisition. INaCl data were recorded digitally on a personal computer using an analog-to-digital converter (ADC-30, CONTEC Microelectronics, San Jose, CA) and sampling at a rate of 1 Hz.

Statistics and data analysis. 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 t-test (18). Differences were considered significant at a level of P < 0.05.
**RESULTS**

**GF109203X blocks and EGTA decreases ANG II-induced loss of NBCe1 cotransporters from the cell surface in *X. laevis* oocytes.** In our previous paper, we showed that ANG II induces a significant decrease in the surface expression of NBCe1 (28). We also reported that ANG II induces a significant decrease in the surface expression of NBCe1 (28). To investigate whether PKC and intracellular Ca$^{2+}$ also play a role in the ANG II-induced decrease in NBCe1 surface expression, we utilized the PKC-specific inhibitor GF109203X and EGTA. Using surface biotinylation followed by Western blot analysis, we determined levels of surface expression of cotransporters in oocytes coexpressing hkNBCe1-EGFP with rat AT1B (see **EXPERIMENTAL PROCEDURES** and Ref. 28). We found that the intensity of the biotinylated NBCe1 band from oocytes treated for 20 min with 1 μM ANG II (Fig. 1A, lane 2) was significantly reduced compared with that of untreated cells (Fig. 1A, lane 1). The intensity of the biotinylated NBCe1 band from oocytes treated for 20 min with 100 nM GF applied together with 1 μM ANG II (Fig. 1A, lane 3) was similar to that of untreated cells (Fig. 1A, lane 1). The intensity of the biotinylated NBCe1 band from oocytes injected with 50 nl of 50 mM EGTA before treatment with 1 μM ANG II (Fig. 1A, lane 4) was less than that of untreated cells (Fig. 1A, lane 1) but greater than that of ANG II-treated cells (Fig. 1A, lane 2). Bars in Fig. 1B show that the normalized intensity of the biotinylated

**Fig. 2**. Confocal images of NBCe1 endocytosis. A: representative single equatorial optical slice of confocal images of a live untreated oocyte expressing NBCe1-EGFP and loaded with 2 μM red FM 4-64 dye. The first panel on left shows brightfield image of outer edge of the oocyte. The second panel from the left shows "red" fluorescence of FM 4-64 styryl dye localized in the plasma membrane of oocyte. The third panel from the left shows "green" fluorescence of NBCe1-EGFP localized within the membrane of the oocyte. Far right panel shows merged image of red and green with yellow, indicating colocalization of FM 4-64 and NBCe1-EGFP within the oocyte membrane. B: representative single equatorial optical slice of confocal images of a live oocyte treated for 20 min with 1 μM ANG II and 2 μM FM 4-64 at room temperature. White arrows in merged field point to endosomal formations sustained with FM 4-64 and NBCe1-EGFP. Yellow indicates colocalization of FM 4-64 (red) and NBCe1-EGFP (green). C, left and middle: representative high-magnification image of endosomal formations in live oocyte treated for 20 min with 1 μM ANG II. Outlined regions (white dotted circles) show colocalization of FM 4-64 and NBCe1-EGFP within endosomal formations budding into the cytosol of the oocyte; right, fluorescence intensity representations of left image showing highest intensities of red and green localized within outlined regions. Scale on far right color-codes low to high intensity of fluorescence. Scale bars = 5 μm. Confocal images were acquired with a Zeiss LSM510 microscope.
Fig. 4. Monensin and ANG II produce a greater inhibition of NBCe1 current than either does alone in voltage-clamped X. laevis oocytes. Voltage-clamp currents were obtained from oocytes coexpressing NBCe1 and AT1. Oocytes were superfused with 5% CO2/33 mM HCO3 solution, voltage clamped to -50 mV for 10 min, and depolarized from -50 to 0 mV to record either control currents before treatment or test currents following treatment (See EXPERIMENTAL PROCEDURES). Values are means ± SD of relative NBCe1 peak currents acquired as the ratio of test to control peak currents in n experiments ([as described earlier (28) and see EXPERIMENTAL PROCEDURES]). Oocytes were untreated (bar A); treated with 1 μM ANG II for 20 min (bar B); *P < 0.005 vs. untreated cells by Student’s t-test; treated with 50 μM monensin overnight (bar C); *P < 0.005 vs. untreated cells by Student’s t-test; and treated with 50 μM monensin overnight followed by 1 μM ANG II for 20 min (bar D); *P < 0.005 vs. ANG II-treated cells by Student’s t-test).

NBCe1 band from oocytes treated with ANG II alone was 39.3 ± 11.5% (n = 6); ANG II together with 100 nM GF was 102.8 ± 25.9% (n = 6); and injected with EGTA and treated with ANG II was 63.0 ± 13.5% (n = 6) of that of untreated cells. Our data suggest that PKC plays a major role in the ANG II-induced decrease in the surface expression of NBCe1. These data also indicate a partial involvement of intracellular Ca2+.

Monitoring endocytosis of NBCe1-EGFP with FM 4-64 fluorescent dye. Here, we investigated the involvement of endocytosis in the ANG II-induced decrease in surface expression of NBCe1. It is known that X. laevis oocytes have machinery for endocytosis, which occurs via plasma membrane vesicles that bud into the cytosol (7, 13, 25, 26, 33). Therefore, to provide evidence for ANG II-stimulated endocytosis of NBCe1, we needed to demonstrate that endosomes which form in ANG II-treated oocytes are stained with EGFP-tagged NBCe1. For this we used confocal fluorescent microscopy in live oocytes coexpressing NBCe1-EGFP with AT1B (See EXPERIMENTAL PROCEDURES). We utilized styryl fluorescent dye FM 4-64, which partitions within the lipid bilayer of the plasma membrane and reversibly stains membranes of endosomes which have been endocytosed (4, 27). We used the brightfield view to position oocytes and visualize the external and internal division (Fig. 2A, far left). Loading oocytes with 2 μM FM 4-64 resulted in strong red dye labeling of the plasma membrane of untreated oocytes (Fig. 2A, second panel from the left). NBCe1-EGFP can be seen localized within the plasma membrane of the oocyte (Fig. 2A, third panel from left). Figure 2A, far right is a merged image of FM 4-64 and NBCe1-GFP showing that both are localized within the plasma membrane.

Next, we treated oocytes for 20 min with 1 μM ANG II, transferred oocytes to dye- and ANG II-free media, and detected colocalization of NBCe1-EGFP and FM 4-64 within vesicular formations that bud into the cytosol (Fig. 2B, white arrows). High-magnification views of these endosomal formations allow one to see the high intensity and colocalization of FM 4-64 and NBCe1-EGFP fluorescence (Fig. 2C, dashed white circles). These data indicate that ANG II induces endocytosis of NBCe1 cotransporters from the cell surface of X. laevis oocytes.

Fig. 5. CaM inhibitor W13 decreases surface expression of NBCe1 and causes endosome-like formations stained with NBCe1-EGFP in X. laevis oocytes. A: typical surface biotinylation study using oocytes coexpressing NBCe1-EGFP and AT1. Biotinylated NBCe1 bands represent the level of surface expression of NBCe1 detected by Western blot analysis using anti-GFP monoclonal antibodies in a dilution of 1:1,000 (see EXPERIMENTAL PROCEDURES). Oocytes were untreated or treated with 100 μM W13 for 50 min. B: bars represent means ± SD of normalized intensity of biotinylated NBCe1 bands acquired as the ratio of intensity of biotinylated NBCe1 bands from W13-treated oocytes to those from untreated control oocytes in 5 experiments. The intensity of the biotinylated NBCe1 bands in oocytes treated with W13 was 34.7 ± 25.7% (n = 5) of that of untreated cells. *P < 0.005 by Student’s t-test. C: representative single equatorial optical slice of confocal images of a live oocyte treated for 50 min with 100 μM W13. Images show fluorescence of NBCe1-EGFP. White arrows in high-magnification image point to endosome-like formations. Scale bars = 5 μm.
Recycling inhibitor monensin significantly reduces the level of surface expression and INBC. Endocytosis can be carried out by increasing internalization of NBCe1 from the plasma membrane or by decreasing recycling of the endocytosed cotransporters back to the plasma membrane (36). Here, we used a recycling inhibitor, the monovalent carboxylic ionophore monensin, to determine whether recycling of the transporter is involved in the ANG II-induced endocytosis of NBCe1. We first performed a surface biotinylation study followed by Western blot analysis to determine whether monensin affects surface expression of NBCe1 in *X. laevis* oocytes (see Experimental Procedures and Ref. 28). Oocytes coexpressing NBCe1-EGFP with AT1B were untreated and treated with 50 μM monensin overnight. A typical experiment shown in Fig. 3A illustrates a significant decrease in the intensity of the surface NBCe1 band in monensin-treated cells. We found that the normalized intensity of the biotinylated NBCe1 band in monensin-treated oocytes was 47.9 ± 7.7% (*n* = 5) of that of untreated control cells (Fig. 3B). To support our biotinylation data, we used confocal fluorescent microscopy in live oocytes to visualize monensin’s effect on NBCe1 surface expression (see Experimental Procedures). Equatorial optical slice images of oocytes revealed that treatment with 50 μM monensin overnight caused endosomal-like formations stained with NBCe1-EGFP (Fig. 3C). In the high-magnification view in Fig. 3C, the white arrow points to a large endosomal formation that buds into the cytosol. Thus our surface biotinylation and confocal microscopy studies revealed that the recycling inhibitor monensin produces a significant loss of NBCe1 cotransporters from the cell surface.

Next, we determined the effect of monensin on NBCe1 functional activity, acquired as INBC in voltage-clamped oocytes coexpressing NBCe1-EGFP with AT1B (see Experimental Procedures and Ref. 28). We found that mean INBC recorded from oocytes treated overnight with 50 μM monensin was 1.4 ± 0.3 μA (*n* = 17) compared with 2.3 ± 0.2 μA (*n* = 17) for untreated control oocytes (Fig. 3D). Thus we detected that INBC was significantly inhibited in oocytes treated with monensin overnight, with the remaining current being 59.7 ± 12.0% (*n* = 17) (Fig. 4, bar C) of that from untreated oocytes. These data show that monensin produces a considerable decrease in the functional activity of NBCe1, probably through significant loss of the cotransporters from the cell surface by inhibiting the recycling of NBCe1 cotransporters back to plasma membrane.

**Applied together, recycling inhibitor monensin and ANG II produce a cumulative inhibitory effect on I**$_{\text{Nbc}}$. We showed above that ANG II induces endocytosis of NBCe1, but we did not clarify whether ANG II induces internalization or inhibits recycling of endocytosed NBCe1 back to the plasma membrane. Monensin, as shown in the above experiments, inhibits recycling of endocytosed NBCe1. To examine whether ANG II and monensin inhibit activity of NBC1 through the same or separate means, we applied ANG II to monensin-treated oocytes. Overnight treatment with 50 μM monensin approximately halves INBC. Twenty-minute treatment with 1 μM ANG II also approximately halves INBC. The remaining currents were 59.7 ± 12.0 (*n* = 17) and 52.4 ± 5.9% (*n* = 7), respectively, of the control current before treatment (bars C and B, respectively, in Fig. 4). In oocytes treated overnight with 50 μM monensin followed by a 20-min application of 1 μM ANG II, INBC was drastically inhibited, with the remaining current being 25.0 ± 8.7% (*n* = 15) of the control (Fig. 4, bar D). This cumulative inhibitory effect indicates that ANG II and monensin decrease functional activity of NBC1 through separate means. Our data suggest that monensin inhibits NBCe1 by blocking the recycling of NBCe1 to the plasma membrane, while ANG II inhibits NBCe1 by inducing internalization of NBCe1.

**Calmodulin inhibitor W13 significantly reduces the level of surface expression of NBCe1.** To help support our results found with monensin, we decided to use another inhibitor of recycling, W13. W13 is known to inhibit the recycling of various transporters in other cells (39) and is also a highly specific CaM antagonist (15). Therefore, we aimed to support our results separating the mechanisms of ANG II and monensin as well as determine the role of CaM in the trafficking of NBCe1. We first set out to determine the effect of W13, as a recycling inhibitor, on the surface expression of NBCe1 in *X. laevis* oocytes. The experiment shown in Fig. 5A represents a typical surface biotinylation experiment in oocytes coexpressing NBCe1-EGFP and AT1 (see Experimental Procedures). A typical Western blot in Fig. 5A shows that a 50-min treatment with 100 μM W13 significantly reduces the level of NBC1 protein at the cell surface. Figure 5B shows that the normalized intensity of the biotinylated NBCe1 bands from oocytes treated with W13 was 34.7 ± 25.7% (*n* = 5) of that of untreated cells. These data indicate that inhibition of CaM produces a significant loss of surface NBCe1 protein, probably through the inhibition of recycling NBC1 to the...
plasma membrane. We used confocal fluorescent microscopy in live cells to visualize W13’s effect on NBCe1 surface expression. Equatorial optical slice images revealed that a 50-min treatment with 100 µM W13 produced endosomal-like formations stained with NBCe1-EGFP and clearly visible at high magnification (Fig. 5C, white arrows). Note that all oocytes also expressed AT1 for control and comparison purposes.

Applied together, W13 with ANG II, but not W13 with monensin, produce a cumulative inhibitory effect on INBC. We determined the effect of W13 on NBC. We pretreated oocytes for 50 min with 100 µM W13. We found that in voltage-clamped oocytes, the remaining \( I_{\text{NBC}} \) was 35.7 ± 6.5% (n = 11) of control current before treatment (Fig. 6, bar C). Note that in oocytes treated with ANG II alone, the remaining INBC was 52.4 ± 5.9% (n = 7) of control (Fig. 6, bar B). Next, we pretreated oocytes for 30 min with 100 µM W13 followed by a 20-min treatment with a mixture of 1 µM ANG II and 100 µM W13. The remaining \( I_{\text{NBC}} \) was 18.7 ± 4.9% (n = 6) of control current before treatment (Fig. 6, bar D). Thus we found that W13 and ANG II applied together produce a much stronger inhibition of \( I_{\text{NBC}} \) than either alone. This cumulative effect suggests that W13 and ANG II inhibit NBCe1 through separate means. Our data and the literature suggest that W13 inhibits NBCe1 by blocking the recycling of NBCe1 to the plasma membrane, while ANG II inhibits NBCe1 by stimulating the internalization of NBCe1. We then provided additional evidence that W13 works as a recycling inhibitor in X. laevis oocytes, as it does in other cells. We applied 50 µM monensin overnight and then 100 µM W13 for 50 min. The remaining \( I_{\text{NBC}} \) was 59.4 ± 8.2% (n = 6) of control (Fig. 6, bar F). Note that monensin treatment resulted in a remaining current of 59.7 ± 12.0% (n = 17) of control (Fig. 6, bar E). W13 and monensin applied together produced an inhibition approximately equal to monensin alone, while W13 alone causes greater inhibition. We speculate that this is due to the action of monensin and the timing of applications. Monensin was applied overnight before W13 application. Once monensin causes its effect on endosomes and recycling is blocked, CaM’s role in controlling NBCe1 recycling may be rendered useless. In summary, lack of a cumulative effect suggests that monensin and W13 affect NBCe1 through similar means. Both monensin and W13 inhibit recycling of NBCe1. Our data suggest that CaM plays a role in the recycling of NBCe1 to the plasma membrane.

CaMKII inhibitor KN93 significantly decreases surface expression and inhibits \( I_{\text{NBC}} \) and when applied together with ANG II produces a cumulative inhibitory effect on \( I_{\text{NBC}} \). The above experiments suggest that CaM is involved in recycling of NBCe1 in X. laevis oocytes. Since CaMKII effects intestinal NBCe1 (2) and CaMKII is a CaM-activated enzyme, we decided to determine the role of CaMKII in NBCe1 surface

**Fig. 7.** CaM kinase II (CaMKII) antagonist KN93, which inhibits surface expression and current of NBCe1, produces an additive effect with ANG II. A: typical surface biotinylation study using oocytes expressing NBCe1-EGFP and AT1. Biotinylated NBCe1 bands represent the level of surface expression of NBCe1 detected by Western blot analysis using anti-GFP monoclonal antibodies in a dilution of 1:1,000 (see EXPERIMENTAL PROCEDURES). Oocytes were untreated or treated for 50 min with 50 µM KN93. B: values are means ± SD of normalized intensity of biotinylated NBCe1 bands acquired as the ratio of intensity of biotinylated NBCe1 band from KN93-treated oocytes to untreated control oocytes in 4 experiments. The intensity of the biotinylated NBCe1 band in oocytes treated with KN93 was 39.8 ± 24.6% (n = 4) that of untreated cells. *P < 0.005 by Student’s t-test. C: voltage-clamp currents were obtained from oocytes coexpressing NBCe1 and AT1. Oocytes were superfused with 5% CO\(_2\)/33 mM HCO\(_3\) solution, voltage clamped to −50 mV for 10 min, and depolarized from −50 to 0 mV to record either control currents before treatment or test currents following treatment (see EXPERIMENTAL PROCEDURES). Values are means ± SD of relative NBCe1 peak currents acquired as the ratio of test to control peak currents in n experiments (see above). Oocytes were untreated (bar A), treated for 20 min with 1 µM ANG II (bar B; *P < 0.005 vs. untreated cells by Student’s t-test); treated with 50 µM KN93 for 50 min (bar C; *P < 0.005 vs. untreated cells by Student’s t-test); and treated by KN93 (50 µM for 30 min) followed by 50 µM KN93 plus 1 µM ANG II for 20 min [bar D; *P < 0.005 vs. cells treated with ANG II and KN93 (separate analysis)].
expression and function. The experiment shown in Fig. 7A represents a typical surface biotinylation study in oocytes coexpressing NBCe1-EGFP and AT1. The Western blot in Fig. 7A shows that a 50-min treatment with 50 μM KN93 significantly reduced the level of NBCe1 protein at the cell surface. Figure 7B shows that the normalized intensity of the biotinylated NBCe1 band from oocytes treated with KN93 was 39.8 ± 24.6% (n = 4) of that of control untreated cells. These data suggest that CaMKII plays a role in controlling surface expression of NBCe1. We determined the effect of KN93 on INBC in voltage-clamped oocytes. We found that a 50-min treatment with 50 μM KN93 caused inhibition of NBCe1 functional activity, with the remaining INBC being 55.0 ± 7.5% (n = 9) (Fig. 7C, bar C) of the control current before treatment. Next, we pretreated oocytes for 30 min with 50 μM KN93, followed by a 20-min treatment with a mixture of 1 μM ANG II and 50 μM KN93. Figure 7C, bar C, shows that the remaining INBC in oocytes treated with a mixture of KN93 and ANG II was 18.1 ± 6.1% (n = 8, bar D) of the control current before treatment. Note that in oocytes treated with ANG II alone, the remaining INBC was 52.4 ± 5.9% (n = 7) of control (Fig. 7C, bar B). These data suggest that inhibition of CaMKII produces a significant decrease in the functional activity of NBCe1 cotransporters. The cumulative effect of ANG II and KN93 together suggests that they work through separate means to inhibit NBCe1. Considering this, and that CaM controls recycling of NBCe1 and that CaMKII is a CaM-activated enzyme, it is very likely that CaMKII plays a role in the recycling of NBCe1 back to the plasma membrane.

**DISCUSSION**

We reported earlier that 1 μM ANG II inhibits current and decreases surface expression of NBCe1 (28). We also reported that ANG II-inhibition of INBC is mediated by PKC and intracellular Ca2+ (28). Binding of ANG II to AT1 receptors causes a cascade of events which eventually activates PKC and increases intracellular Ca2+ concentration (11). The present report shows that PKC plays a major role and intracellular Ca2+ plays some role in the ANG II-induced loss of NBCe1 from the cell surface. This conclusion is based on the demonstration that GF109203X, which specifically inhibits all PKC isoforms, applied together with ANG II completely prevents the loss of NBCe1 from the cell surface (Fig. 1A, lane 3, Fig. 1B, bar 3). Furthermore, chelating intracellular Ca2+ with EGTA in oocytes treated with ANG II reduces the amount of NBCe1 lost from ANG II application alone (Fig. 1A, lane 4, Fig. 1B, bar 4).

Endocytosis is a common mechanism responsible for the loss of surface expression of several membrane proteins (35). Our findings indicate that endocytosis also regulates surface expression of NBCe1 in ANG II-treated oocytes. To show this, we utilized FM 4-64, an impermeant styryl dye, which fluoresces exclusively in a lipid environment of either plasma or endosomal membranes (4). We used FM 4-64 as a plasmalemma marker in nontreated oocytes when dye was present in the media and as an endocytotic marker when dye was washed from the media. By confocal microscopy in live cells, we showed that NBCe1 localized at the plasma membrane of oocytes and colocalized with FM 4-64 in endosomal formations after ANG II treatment (Fig. 2).

We demonstrated that ANG II-induced endocytosis is not a “global” event involving all membrane proteins in X. laevis oocytes. For this we used another membrane transporter, an excitatory amino acid transporter, EAAT3, coexpressed with AT1B in X. laevis oocytes (the EAAT3-EGFP construct was a kind gift of Drs. Thomas S. Otis and Meera Pratap, University of California, Los Angeles, CA). Using confocal microscopy, no loss of surface expression of EAAT3-EGFP was observed after a 20-min 1 μM ANG II treatment, with normalized fluorescent intensity of total plasma membrane region being

Fig. 8. Putative model of the membrane trafficking of NBCe1 in X. laevis oocytes. In internalization from the plasma membrane, ANG II binds to the AT1 receptor, activating PKC and raising intracellular Ca2+. ANG II-activated PKC and Ca2+ induce endocytosis of NBCe1. Endocytosis can be carried out by increasing internalization of NBCe1 or by decreasing recycling of endocytosed cotransporters (36). ANG II induces the internalization of NBCe1 and does not change constitutive recycling of the cotransporter, causing loss of NBCe1 at the surface and inhibition of the cotransporter activity. In oocytes treated with GF109203X or EGTA, the ANG II-induced internalization of NBCe1 was prevented. In recycling back to the plasma membrane, CaM plays an important role in the recycling pathway but not in endocytosis (16, 17, 22, 24). CaM and CaMKII control recycling of NBCe1. CaM/CaMKII antagonists W13 and KN93 inhibit NBCe1 recycling, causing loss of surface expression and activity of the cotransporter. The recycling inhibitor monensin also reduces recycling of NBCe1 back to the plasma membrane, causing loss of surface expression and activity of the cotransporter.
\[ 101 \pm 13\% \ (n = 17) \text{ of that of untreated oocytes (data not shown). In parallel experiments, similar treatment resulted in a significant loss of surface expression of NBCe1-EGFP, with normalized fluorescent intensity of total plasma membrane region being 56.2 \pm 12.8\% \ (n = 7) \text{ of that of untreated oocytes (data not shown). These data suggest that angi II-induced endocytosis of NBCe1 is not global and may be specific for the NBCe1 cotransporter under conditions of our experiments.}

The loss of surface NBCe1 could be carried out through increasing internalization of or by inhibiting the recycling of constitutively endocytosed cotransporters (35). To investigate the mechanism of angi II-stimulated endocytosis of NBCe1, we required a means of blocking recycling of NBCe1 separate from angi II. To do this, we used the recycling inhibitor monensin (3, 37, 38, 40). Monensin caused a significant loss of NBCe1 cotransporters from the cell surface (Fig. 3, A and B) and induced the appearance of large endosome-like vesicles stained with NBCe1-EGFP (Fig. 3C, white arrow). Monensin also inhibited \( I_{\text{NBC}} \) in voltage-clamped oocytes (Figs. 3D and 4, bar C). When applied together, angi II and monensin resulted in a cumulative inhibitory effect on \( I_{\text{NBC}} \) (Fig. 4, bar D). This suggests that monensin and angi II work through separate means to inhibit NBCe1. Thus we showed that angi II most likely induces internalization of NBCe1 from the plasma membrane. However, our data are not able to differentiate between angi II causing additional internalization and that causing an increase in the rate of constitutive internalization without affecting the rate of recycling back to the membrane. It will be of great interest to determine in mammalian renal cells the rates of constitutive and stimulated endocytosis of NBCe1 and to identify what mechanisms are involved (clathrin, caveolae, etc.).

It has been shown that recycling of assorted endocytosed transporters back to the membrane is dependent on CaM (1, 9, 14, 23). It has been reported by others that CaM plays a significant role in the regulation of functional activity of NBCe1 in various cells (2, 10, 31, 32). However, nothing was known about the CaM's role in the control of NBCe1 surface expression, nor CaM’s effects of NBCe1 in X. laevis oocytes.

The present report shows that CaM plays a role in the recycling of endocytosed NBCe1 back to the plasma membrane. This conclusion is based on the demonstration that inhibiting CaM with W13 resulted in removal of the cotransporter from the cell surface (Fig. 5, A and B). W13 induces the appearance of endosome-like vesicles stained with NBCe1-EGFP (Fig. 5C, white arrows). W13 also significantly inhibited \( I_{\text{NBC}} \) in voltage-clamped oocytes (Fig. 6, bar C). Moreover, when applied together angi II and W13 resulted in a cumulative inhibitory effect on \( I_{\text{NBC}} \) (Fig. 6, bar D). In contrast, when monensin and W13 were applied together there was no cumulative effect (Fig. 6, bar F).

The CaM-dependent enzyme CaMKII has been reported to affect NBCe1 function (2). Therefore, we aimed to define the role of CaMKII in the regulation of NBCe1 trafficking. We showed that inhibiting CaMKII with KN93 results in a significant loss of NBCe1 from the cell surface (Fig. 7, A and B) and in inhibition of \( I_{\text{NBC}} \) (Fig. 7C, bar C). When applied together, angi II and KN93 produce a cumulative effect on \( I_{\text{NBC}} \) (Fig. 7C, bar D). This suggests that these two work through separate means to inhibit NBCe1. Knowing that CaMKII is a CaM-activated enzyme and by showing that CaM is involved in the recycling of NBCe1 back to the plasma membrane, it is likely that CaM works through CaMKII to regulate the recycling process of NBCe1.

Our results show that the inhibitory effect of W13 on NBCe1 is faster and more robust than monensin’s effect (Figs. 3B, 5B, and Fig. 6, bars C and E). The observed differences may be attributed to possibly different mechanisms behind W13- and monensin-inhibition of NBCe1 recycling. Since these mechanisms are still not well understood, we can only speculate about the reasons behind the observed differences in action of these two compounds. It has been shown that calmodulin antagonists and monensin can raise endosomal pH, which causes a major disruption of the recycling pathway (1, 3, 37, 40). However, some have suggested that monensin is ineffective in mildly acidic early endosomes, and thus recycling may be incompletely blocked by monensin (22). Additionally, it has been suggested that CaM is involved in various portions of the recycling pathway, which also may explain the stronger and faster effect of W13 (16, 17, 22, 24).

A model proposed from our data is summarized in Fig. 8. Our findings suggest that 1) acute application of high concentration of angi II induces endocytosis of renal NBCe1 expressed in X. laevis oocytes; 2) angi II-induced endocytosis of NBCe1 is mediated mainly by PKC and in part by intracellular Ca\(^{2+}\); 3) CaM and CaMKII control surface expression and functional activity of NBCe1 in the absence of induced endocytosis; and 4) angi II and CaM/CaMKII regulate NBCe1 via separate mechanisms: angi II induces internalization of NBCe1, while CaM/CaMKII controls recycling of the endocytosed transporters back to the cell surface.

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GRANTS

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