Calcium-sensing receptor signaling pathways in medullary thick ascending limb cells mediate COX-2-derived PGE₂ production: functional significance

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Abdullah HI, Pedraza PL, McGiff JC, Ferreri NR. Calcium-sensing receptor signaling pathways in medullary thick ascending limb cells mediate COX-2-derived PGE₂ production: functional significance. Am J Physiol Renal Physiol 295: F1082–F1089, 2008. First published August 6, 2008; doi:10.1152/ajprenal.90316.2008.—We determined the functional implications of calcium-sensing receptor (CaR)-dependent, Gₛ- and Gₛₕ-coupled signaling cascades, which work in a coordinated manner to regulate activity of nuclear factor of activated T cells and tumor necrosis factor (TNF)-mediated COX-2 expression and PGE₂ synthesis. We tested the hypothesis that these pathways contribute to the effects of CaR activation on ion transport in mTAL cells. Ouabain-sensitive O₂ consumption, an in vitro correlate of ion transport in the mTAL, was inhibited by ~70% in cells treated for 6 h with extracellular Ca²⁺ (1.2 mM), an effect prevented in mTAL cells transiently transfected with a dominant negative CaR over-
Reagents. Tissue culture media were obtained from Life Technologies (Grand Island, NY). Reagent-grade chemicals and collagenase (type 1A) were from Sigma (St. Louis, MO). Polyvinylidene difluoride (PVDF) membranes were obtained from Amersham (Arlington Heights, IL). The phosphoinositid phospholipase C (PI-PLC) inhibitor, U73122, and inactive analog, U73343, as well as pertussis toxin (PTX), GF 109203X, and NS-398 were purchased from BIOMOL; cyclosporin A (CsA), VIVIT, and the PTX B-Oligomer were purchased from Calbiochem. Preliminary experiments were performed to establish the optimal conditions for each inhibitor with respect to concentration and time.

Isolation of mTAL cells. The isolation and characterization of mTAL cells (~95% purity) were performed as previously described (12, 19). In brief, male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.65 mg/100 g body wt). The kidneys were perfused with sterile 0.9% saline, via retrograde perfusion of the aorta, and cut along the corticopapillary axis. The inner stripe of the outer medulla was excised, minced with a sterile blade, and incubated for 10 min at 37°C in a 0.1% collagenase solution gassed with 95% oxygen. The suspension was sedimented on ice, mixed with HBSS containing 2% BSA, and the supernatant containing the crude suspension of tubules was collected. The collagen digestion was repeated three times with the remaining undigested tissue. The combined supernatants were centrifuged for 10 min, resuspended in HBSS, and filtered through a 52-μm nylon mesh membrane (Fisher Scientific, Springfield, NJ). The filtrated solution was discarded, and the tubules retained on the mesh were resuspended in HBSS. Then, the solution was centrifuged at 500 rpm for 10 min, supernatant was aspirated, and the cells were cultured in renal epithelial cell basal medium (REBM, Cambrex), containing renal epithelial cell growth medium (REGM, Cambrex) consisting of rhEGF, insulin, hydrocortisone, GA-1000 (gentamycin sulfate and amphotericin B), 0.5% FBS, epinephrine, triiodothyronine, and transferrin. The combined supernatants were centrifuged for 10 min, supernatant was aspirated, and the cells were cultured in renal epithelial cell basal medium (REBM, Cambrex), containing renal epithelial cell growth medium (REGM, Cambrex) consisting of rhEGF, insulin, hydrocortisone, GA-1000 (gentamycin sulfate and amphotericin B), 0.5% FBS, epinephrine, triiodothyronine, and transferrin. After 3–4 days, monolayers of cells were 80–90% confluent. The cells were quiesced for 24 h in RPMI containing 0.42 mM CaCl2 and 0.5% FBS, 1-glutamine (2 mM), 100 U/ml streptomycin-penicillin (GIBCO), MEM nonessential amino acids (GIBCO), MEM sodium pyruvate, and β-mercaptoethanol before their use. In all experiments, “control conditions” (i.e., no addition of CaCl2) reflects that cells were incubated in media containing 0.42 mM Ca2+. This amount of Ca2+ should be added to the amounts used to challenge the cells to obtain total Ca2+ present. These control conditions were selected based on previous work showing that the CaR is functionally insensitive when Ca2+ concentrations are <1 mM (62).

Western blot analysis. Cells were pretreated with U73122 (500 nM), the synthetic peptide VIVIT (20 μM), CsA (0.55 ng/ml), PTX (100 ng/ml), or GF 109203X (20 nM) for 15 min-1 h before incubation of cells with or without 1.2 mM CaCl2 for 6 h. The quiescence media was removed and cells were washed three times with cold PBS, scraped, and collected with 200 μl/well of lysis buffer (100 mM Tris-HCl, pH 7.6, 5 mM EDTA, 1% SDS, and 1 mM PMSF added fresh). Protein concentrations were determined using a detergent-compatible Bio-Rad protein assay kit. Cell lysates were normalized according to protein concentration with the same lysis buffer; and aliquots of protein were removed and cells were placed in 1 ml of serum-free OPTI-MEM. Next, membranes were incubated with rabbit anti-mouse COX-2 polyclonal antibody (1:1,000; Cayman), diluted in 1% nonfat dry milk with 0.1% Tween 20, at room temperature. Membranes were washed three times with 1× TBS-Tween or PBS-Tween and then incubated in goat anti-rabbit IgG-horseradish peroxidase (1:5,000; Santa Cruz Biotechnology) for 30 min. Finally, membranes were washed three times and developed with the Amersham ECL Western blotting detection reagents and exposed on Kodak Biomax Light Film (Fisher).

Measurement of PGE2. Primary cultured rat mTAL cells were quiesced overnight and then incubated with or without 1.2 mM CaCl2 for 6 h at 37°C/5% CO2. PGE2 levels in cell-free supernatants were determined by ELISA (Cayman), according to the protocol provided by the manufacturer and as previously reported (57).

Transient transfection. mTAL cells were cultured to 70–80% confluence and transfected as described (55). In brief, the medium was removed and cells were placed in 1 ml of serum-free OPTI-MEM medium containing 7 μg/well of either plasmid DNA containing a dominant negative R796W CaR constructor empty plasmid vector (pcDNA3.1) and 10 μl lipofectamine reagent (Life Technologies) for 4 h at 37°C/5% CO2. The medium was then removed, and cells were cultured for an additional 12 h in REGM containing 0.5% FBS. The cells were quiesced overnight in RPMI medium containing 0.5% FBS and then treated with the appropriate reagents for the indicated times. Transfection efficiency was evaluated as described (1).

Measurement of oxygen consumption. Oxygen consumption was measured as described (17). In brief, quiescence medium (RPMI 1640 containing 0.5% FBS) was aspirated and replaced with 1 ml/well dispase (BD Biosciences) and incubated for 45 min at 37°C/5% CO2. Cells were then collected into a plastic centrifuge tube, centrifuged at 500 rpm for 10 min, and washed three times to remove remaining dispase. The final cell density was adjusted to contain 25,000 cells in 100 μl and oxygen consumption was determined in 96-well round bottom plates coated with a proprietary biosensor, which fluoresces when oxygen is depleted (BD Biosciences). The plates were read on a Bio-Tek FLx 800 fluorescence microplate reader, set to read the bottom of the plate with excitation/emission wavelengths at 485/620 nm; octuplet fluorescence readings were converted to oxygen consumption rates using the KC4 (Kineticalc) program according to the manufacturer’s protocol. Ouabain-sensitive O2 consumption is an index of Na+-K+-ATPase activity in mTAL cells and was determined by taking the difference between cells incubated without (total oxygen consumption) or with ouabain (1 mM). All inhibitors used in the study also were tested in the absence or presence of ouabain.

Na+-K+-ATPase activity. The assay is based on the hydrolysis of ATP in the presence and absence of ouabain (15). Total phosphate release was determined using a microplate assay using the Biomol Biolumin green reagent. The reaction mixture consists of (in mM) 37.5 imidazole, 75 NaCl, 5 KCl, 1.0 EGTA, 5 MgCl2, 6 NaN3, and 75 Tris-HCl (solution A) and 4 mM ATP. To assess ouabain-insensitive phosphate release, solution A was modified to contain 1 mM ouabain and 150 mM Tris, and no NaCl or KCl (solution B). Confluent cultures in six-well plates were washed two times with scavenging buffer (300 mM mannitol, 10 mM Tris, 10 mM HEPES, pH 7.4) and then harvested and centrifuged at 5,000 rpm for 5 min. The pellet was resuspended in a 1:10 dilution of solution B and subjected to three cycles of freeze-thaw; mTAL cells concentration was determined. Aliquots of protein were preincubated for 10 min at 37°C in solution A or B. The reaction was initiated by addition of 4 mM ATP and continued for 5 min at 37°C. The reaction was stopped by rapidly transferring the tubes to an ice-cold water bath, followed by the addition of 50 μl of 50% ice-cold TCA. After 10 min, the reaction mixture was centrifuged at 10,000 rpm and the supernatant was diluted 1:20 for determination of inorganic phosphate. An aliquot (50 μl) of the reaction mixture was incubated in a 96-well plate with 100 μl of Biolum green reagent for 20–30 min at room temperature; absorbance at 620 nm was determined. A standard curve was constructed using a potassium phosphate solution supplied by the manufacturer. The reactions were carried out under conditions that enabled measurements to fall within the linear part of the standard curve. Biolum green is a proprietary formulation of Malachite green made by Biolum for phosphate determinations.
**RESULTS**

**CaR-mediated activation of Gq and Gi increases COX-2 expression and PGE2 synthesis in mTAL cells.** Inhibition of Gq with U73122, a PI-PLC inhibitor, and Gi with PTX revealed the extent to which these pathways contribute to CaR-mediated COX-2 expression in mTAL cells. Cells were quiesced overnight and then preincubated for 15 min, with or without U73122 (500 nM), before addition of Ca2+ (1.2 mM) for 6 h. Pretreatment with U73122 abolished Ca2+-mediated increases in COX-2 protein expression indicating that PI-PLC contributes to COX-2 expression in mTAL cells (Fig. 1A). Similarly, cells were preincubated for 1 h, with or without PTX (100 ng/ml) or the PTX B-oligomer (enzymatically inactive subunit; 100 ng/ml), before Ca2+ stimulation. PTX abolished Ca2+-mediated increases in COX-2 protein expression (Fig. 1B). In contrast, COX-2 expression was not altered in cells challenged with Ca2+ following preincubation with PTX B-oligomer suggesting that the enzymatically active A-protomer in PTX, which is involved in ADP ribosylation of the cysteine moiety on the alpha subunit of Gαi, was responsible for the inhibitory effect of PTX (Fig. 1B).

We reported that CaR-mediated PGE2 synthesis in mTAL cells is COX-2 dependent (55). Since Gq and Gi play major roles in the regulation of COX-2 expression in these cells, we tested the role of the two G proteins on PGE2 synthesis, following CaR activation. Addition of Ca2+ increased PGE2 synthesis that was inhibited by pretreatment with the U73122, but not the inactive analog U73343 (Fig. 1C). Neither basal PGE2 synthesis nor cell viability was altered by these compounds. Similarly, pretreatment for 1 h with PTX, but not the inactive B-oligomer, inhibited CaR-mediated PGE2 synthesis, but had no effect on basal PGE2 synthesis (Fig. 1D). Collectively, these data suggest that Gq- and Gi-coupled signaling initiated by CaR activation is required for COX-2-derived PGE2 production by mTAL cells.

**CaR-mediated COX-2 expression and PGE2 synthesis are CaN and NFAT dependent.** Previous work demonstrated that COX-2 expression in the outer medulla of the kidney is CaN dependent; however, the cell type in which this occurred was not identified (30). We determined whether CaN/NFAT signaling mediates CaR-mediated COX-2 expression in mTAL cells. Application of CsA, which forms a complex with cyclophilin and subsequently inhibits CaN phosphatase activity by...
directly binding the CaN-A subunit of CaN (39), was used to determine whether CaN is required for CaR-mediated increases in COX-2 expression and PGE_2 synthesis. Indeed, pretreatment with CsA (0.55 ng/ml) abolished Ca^{2+}-mediated increases in COX-2 expression and PGE_2 synthesis (Fig. 2, A and B). A role for CaN in producing COX-2 expression and PGE_2 synthesis in mTAL cells was also supported by selective blockade of the CaN binding site for NFAT with a high-affinity peptide, VIVIT (20 μM), which prevented CaR-mediated COX-2 expression and PGE_2 synthesis (Fig. 2, A and B). These findings indicate that a CaN/NFAT interaction regulates COX-2 expression and PGE_2 synthesis in mTAL cells.

**Activation of the CaR inhibits ouabain-sensitive O_2 consumption.** As a large component of total O_2 consumption in mTAL cells is committed to the transport of Na^+, determination of ouabain-sensitive O_2 consumption was used to screen for alterations in ion transport function in response to CaR activation. This method is a useful index of Na^+ transport in renal epithelial cells. Basically, increased O_2 consumption by the mTAL indicates increased transport by this segment, whereas a decline in O_2 consumption indicates decreased transport that can be reversed by inhibiting CaR signaling pathways (13, 54). We previously showed using a Clark-type electrode that ouabain (1 mM), an inhibitor of the basolateral Na^+-K^+-ATPase, and bumetanide, a selective inhibitor of the apical Na^+-K^+-2Cl^– cotransporter (NKCC2), inhibited ouabain-sensitive O_2 consumption by ~40–60% in mTAL cells (19); similar results were obtained using the present detection method. CaR-mediated inhibition of ouabain-sensitive O_2 consumption in mTAL cells was prevented when cells were transiently transfected with a dominant negative (R796W) CaR construct, whereas transfection with an empty vector (pcDNA3.1) was without effect on the inhibitory action of Ca^2+ on O_2 consumption (Fig. 3). Therefore, Ca^{2+}-mediated decreases in ouabain-sensitive O_2 consumption appear to be a specific function of CaR activation.

An inhibitory effect on ouabain-sensitive O_2 consumption in mTAL cells reflects either a direct effect on Na^+-K^+-ATPase activity or secondary effect on Na^+ pump activity due to inhibition at sites that regulate Na^+ entry. Accordingly, Na^+-K^+-ATPase activity was measured to determine whether CaR activation in mTAL cells affects Na^+ influx or efflux. Cells were exposed to Ca^{2+} for 6 h, protein concentration was determined, and inorganic phosphate was measured in samples optimized for enzyme kinetics using Biomol green (Biomol). Na^+-K^+-ATPase activity (ouabain-sensitive fraction) was determined under V_{max} conditions and calculated as the difference between total ATPase (assayed in the absence of ouabain) and ouabain-resistant ATPase (assayed in the presence of 1 mM ouabain). CaR activation did not affect Na^+-K^+-ATPase activity in mTAL cells (control: 0.43 ± 0.2; Ca^{2+}: 0.41 ± 0.15 nmol P_i min^{-1} μg protein^{-1}) suggesting that inhibition of ouabain-sensitive O_2 consumption reflects a decrease in pump activity that is secondary to inhibition of Na^+ entry at the apical membrane. Determinations were then made in cells treated with bumetanide and amiloride, to block Na^+ entry via the NKCC2 and Na^+–H^+ exchanger pathways, respectively. Bumetanide (50–200 μM) inhibited O_2 consumption in a concentration-dependent manner [control: 130 ± 2.2 vs. bumetanide (100 μM): 81 ± 3.6 pmol O_2/min/2.5 × 10^4 cells], and slightly greater inhibition was apparent when amiloride was added together with bumetanide (−45–50%). The 40% inhibition in O_2 consumption in response to bumetanide (100 μM) is similar to inhibition of cotransporter activity determined in mTAL cells using ^86^Rb uptake (19, 35). CaR activation did not inhibit O_2 consumption in the presence of bumetanide/amiloride. These findings support the interpretation that CaR activation inhibits NKCC2 activity and additionally that the decrease in ouabain-sensitive O_2 consumption occurs subsequent to inhibition of Na^+ influx.

CaR activation decreases O_2 consumption via COX-2. Secondary inhibition of Na^+-K^+-ATPase activity in the mTAL, subsequent to inhibition of apical K^+ channel and NKCC2 activity by PGE_2, is the basis for decreased ouabain-sensitive O_2 consumption (32, 37). As CaR activation increases COX-2 expression and PGE_2 synthesis in mTAL cells, the effects of COX-2 inhibition on CaR-mediated ouabain-sensitive O_2 consumption were determined. Activation of the CaR inhibited O_2 consumption by ~70% (Fig. 4). Pretreatment for 30 min with the selective COX-2 inhibitor NS-398 (1 μM) partially reversed CaR-mediated decreases in O_2 consumption, but did not alter basal level O_2 consumption (Fig. 4). Thus, the effects of CaR activation on mTAL O_2 consumption appear to involve a COX-2-dependent component. These data indicate that CaR
activation and COX-2 expression contribute to a mechanism that affects ion transport in mTAL cells.

**Identification of signaling pathways that regulate ouabain-sensitive O2 consumption in mTAL cells.** We hypothesized that inhibition of each of the pathways involved in CaR-mediated COX-2-derived PGE2 synthesis should also prevent CaR-mediated decreases in ouabain-sensitive O2 consumption. Inhibition of signaling via Gq, by pretreatment for 15 min with U73122, and Gi, by pretreatment for 1 h with PTX, abolished Ca2+ -mediated decrease in ouabain-sensitive O2 consumption was prevented in cells transfected with the inactive CaR mutant, R796W, but not pcDNA3.1 empty vector; n = 3.

We showed that CaR activation increases PKC activity in mTAL cells, which contributes to COX-2-derived PGE2 synthesis (57). Inhibition of PKC activity by pretreatment with GF 1086 X (20 nM) for 15 min before addition of Ca2+ partially reversed CaR-mediated decreases in O2 consumption, suggesting that PKC contributes to the regulation of mTAL transport function (Fig. 6). Moreover, an increase in PKC activity was also shown to be essential to Gq- and Gi-mediated stimulation of CaN activity (1, 2). Accordingly, as we showed that the CaR utilizes a CaN-NFAT-dependent mechanism to induce COX-2-derived PGE2 synthesis, we assessed mTAL O2 consumption in the absence and presence of CsA and VIVIT. CsA (0.55 ng/ml) reversed CaR-mediated decreases in O2 consumption by ~50%, while the selective CaN/NFAT peptide inhibitor, VIVIT, reversed the Ca2+ -mediated decrease in ouabain-sensitive O2 consumption (Fig. 7). Thus, a CaN-NFAT interaction also presumably regulates CaR-mediated ion transport function in the mTAL.

**DISCUSSION**

We demonstrated that Gq- and Gi-coupled CaR signaling pathways mediated COX-2-derived PGE2 synthesis and that each pathway regulates mTAL ion transport. We found that inhibition of CaR signaling via PI-PLC, PKC, CaN/NFAT, or
of selected activating mutations of the CaR in which patients with Bartter syndrome present with salt and Ca\(^{2+}\) wasting due to reduced ion transport in the TAL affected by PGE\(_2\) and explains the beneficial effects of nonsteroidal anti-inflammatory drugs in reducing the natriuresis, hypercalciuria, and polyuria of Bartter syndrome (28, 41). The inhibitory effects of CaR activation on ouabain-sensitive O\(_2\) consumption that \(\mathrm{Ca}^{2+}/\mathrm{H}^{11001}\) and inhibited PGE\(_2\)-derived prostaglandins (PGE\(_2\)) and ouabain-sensitive O\(_2\) consumption via COX-2, in the absence of a direct effect on Na\(^{+}/\mathrm{K}^{+}\)-ATPase activity in mTAL cells, suggest that CaR activation inhibits Na\(^{+}\) entry in these cells. The transport of NaCl across the apical membrane of the TAL occurs mainly via the bumetanide-sensitive NKCC2 (22, 31). The reabsorption of NaCl is then completed, as Na\(^{+}\) exits the cell via the basolateral Na\(^{+}/\mathrm{K}^{+}\)-ATPase, while Cl\(^{-}\) diffuses along its electrochemical gradient through basolateral KCl cotransporters or Cl\(^{-}\) channels. However, the data do not rule out the possibility that CaR activation affects mTAL transport by more than one mechanism as the apical K\(^{+}\) channel is a major contributor to K\(^{+}\) recycling, which is essential for maintenance of NKCC2 activity (23, 26). As indicated, TAL function is subject to both short- and long-term regulatory mechanisms. For instance, long-term increases in circulating vasopressin levels increase expression of the NKCC2 and transepithelial chloride transport in the TAL (5, 33). Short-term regulation of NKCC2 has also been observed and involves effects on both activity and trafficking (18, 24, 43, 44, 47). CYP450- and COX-derived metabolites of arachidonic acid (AA) inhibit ion transport in the mTAL by several different mechanisms including interactions with apical K\(^{+}\) channels and NKCC2 (32, 37, 38, 52, 53). Thus, it is important to understand how molecules that act in the TAL affect AA metabolism since regulation of ion transport in this segment may occur in response to signals induced by receptor activation (20, 21, 58, 60). Indeed, PGE\(_2\) inhibits apical K\(^{+}\) channel, as well as NKCC2 activity, in murine mTAL cells, the latter by a mechanism that is not secondary to inhibition of Na\(^{+}/\mathrm{K}^{+}\)-ATPase (32, 37).

The epidemiological literature indicates an inverse relationship between Ca\(^{2+}\) intake and BP; individuals with the lowest Ca\(^{2+}\) intake (<700 mg/day) had the highest BP. Increased Ca\(^{2+}\) intake decreases BP in several experimental models of...
hypertension; however, clinical studies based on patients with higher baseline Ca\textsuperscript{2+} levels may fail to detect an effect of Ca\textsuperscript{2+} supplementation on BP because of a ceiling effect that is observed for individuals consuming a diet containing more than ~700 mg/day (6, 40). We showed that the CaR activated NFAT to increase PGE\textsubscript{2} synthesis via a mechanism involving TNF production and COX-2 expression in the mTAL and contributing to inhibition of ouabain-sensitive O\textsubscript{2} consumption, an in vitro correlate of natriuresis in the mTAL. Interestingly, dietary Ca\textsuperscript{2+} supplementation prevented NaCl-dependent increases in BP by prompting an alteration in renal function; namely, the rapid adjustment of extracellular fluid volume via diuresis and natriuresis (6, 46). These studies indicate that high Ca\textsuperscript{2+} intake facilitates salt and water excretion consistent with the inhibitory effects on Na\textsuperscript{+} transport in response to the CaR/TNF/COX-2 pathway that operates in the mTAL. Also, consistent with this role of Ca\textsuperscript{2+} intake on salt excretion are the experiments in which a dominant negative CaR (R796W) overexpressed in mTAL cells prevented the inhibitory effects of CaR activation on ouabain-sensitive O\textsubscript{2} consumption. These in vitro experiments support those studies indicating that persons with inactivating CaR mutations exhibit an impaired ability to excrete Ca\textsuperscript{2+} in response to hypercalcaemia, while individuals with activating CaR mutations had excessively high urinary Ca\textsuperscript{2+} excretion, thus supporting a significant role for the CaR in affecting salt and water excretion (3, 6, 10, 27).

Furthermore, hypercalcaemia in rats produced polyuria and natriuresis associated with decreased expression of the NKCC2 and ROMK in the inner stripe of the outer medulla (36, 61). Interestingly, vasopressin increased NKCC2 expression, as well as NaCl reabsorption (33). Vasopressin regulates the countercurrent multiplication system and is modulated by increases in cAMP and PKC activity. Decreased responsiveness of the kidney to vasopressin appears to be an important cause of polyuria in hypercalcaemia associated with changes in adenylate cyclase and PKC activities (4, 11, 25, 34, 45). Thus, CaR-mediated G\textsubscript{q} and G\textsubscript{i}-coupled signaling that inhibits transporter function in a PGE\textsubscript{2}-dependent manner may counteract the effects of vasopressin in the mTAL, a mechanism that likely acts in concert with the CaR-mediated antagonism of AVP-dependent water permeability in the collecting duct (51).

We showed that the CaR signals in a G\textsubscript{q} and G\textsubscript{i}-dependent manner that is CaN/NFAT mediated to increase PGE\textsubscript{2} synthesis via a TNF-dependent manner (2). We conclude that an autocrine mechanism involving detection of extracellular Ca\textsuperscript{2+} in the mTAL regulates salt and water balance initiated by CaR-mediated COX-2-derived PGE\textsubscript{2} production correlated with greatly diminished ouabain-sensitive O\textsubscript{2} consumption.

**GRANTS**

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