JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2000, 51, 4, 587—595

D. WANG, J. C. MCGIFF, N. R. FERRERI[1]

REGULATION OF CYCLOOXYGENASE ISOFORMS IN THE RENAL THICK ASCENDING LIMB: EFFECTS OF EXTRACELLULAR CALCIUM

Department of Pharmacology, New York Medical College, Valhalla, New York 10595, USA

We previously showed that primary cultures of mTAL cells express cyclooxygenase 2 (COX-2) when challenged with tumor necrosis factor alpha (TNFα) or phorbol myristate acetate (PMA). Moreover, expression of COX-2 was linked to decreases in TNFα-mediated ^{86}Rb uptake, an *in vitro* correlate of natriuresis. mTAL cells in primary culture express calcium sensing receptor (CaR), a G-protein coupled receptor that senses changes in extracellular calcium concentration and ultimately increases intracellular calcium concentration ($[Ca^{2+}]_i$) and protein kinase C (PKC) activity. PGE_2 synthesis by mTAL cells increases in a dose- and time-dependent manner after exposure of these cells to extracellular Ca^{2+}. Similar effects were observed when cells were challenged with the CaR-selective agonist, poly-L-arginine. These data suggest that intracellular signaling mechanisms initiated *via* activation of CaR contribute to mTAL PGE_2 synthesis. As TNF production is calcium-sensitive in some cells types, we postulate that these effects involve the regulation of COX-2 expression *via* a TNF-dependent mechanism. The functional implications of these studies relate to a cytokine-mediated mechanism that contributes to salt and water balance, and suggests that small changes in Ca^{2+}_o may contribute to the regulation of these events. The possibility that the effects of Ca^{2+}_o involve activation of CaR suggests that novel calcimimetic molecules might be useful in conditions, such as hypertension or other conditions, in which manipulation of extracellular fluid volume provides beneficial effects.

Key words: *cyclooxygenase-2, kidney, calcium-sensing receptor, tumor necrosis factor-alpha*

INTRODUCTION

There are two isoforms of prostaglandin H (PGH) synthase: commonly referred to as cyclooxygenase-1 (COX-1) and COX-2, respectively. These two enzymes catalyze the same reactions; i.e., arachidonic acid (AA) is converted to PGG_2 *via* the COX reaction, followed by a peroxidase reaction in which the 15-hydroperoxyl group of PGG_2 is reduced to the 15-hydroxyl group of PGH_2. Expression of COX-2 appears to be differentially regulated in the kidney. For instance, recent studies have shown that COX isoforms are regulated in a zone-specific way by dietary salt (1) and suggest that COX-2 may subserve different functions in the cortex (vascular resistance) and medulla (salt and water homeostasis). Recent work from our laboratory has shown that the TNF-mediated decrease in rubidium uptake, an *in vitro* correlate of natriuresis, by primary cultures of mTAL cells is COX-2 dependent (2). In addition, COX-2 expression by specific renal structures depends on the nature of the inducing stimulus. Macula densa cells expressed COX-2 when rats were placed on a low sodium diet for seven days (3), while adrenalectomy (AdrX) revealed a substantial recruitment of TAL cells containing COX-2 from the cortex towards the medulla (4). As AdrX has been associated with a decrease in sodium reabsorption by the loop of Henle (5), the preferential expression of COX-2 in the cortex rather than the medulla after AdrX may reflect a functional adaptation to conserve salt in the absence of aldosterone. COX-2 expression in the mTAL also may provide a mechanism that contributes to the regulation of renal function in a time-dependent manner. Thus, short-term regulation of ion transport in the mTAL by angiotensin II (Ang II) occurs in a COX-independent manner, while long-term regulation was TNF- and COX-2 dependent (*Fig. 1*) (2, 6). Interestingly, short-term incubations with AA

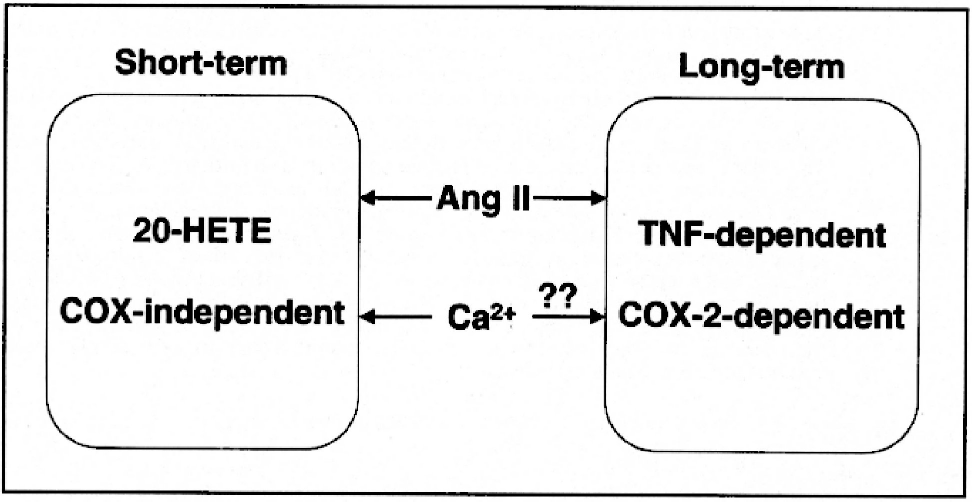

Fig. 1. Short- and long-term regulatory mechanisms in the mTAL. Ang II-mediated short-term (<15 min) regulatory mechanisms that affect ion transport in the mTAL are 20-HETE-dependent and COX-independent. In contrast, long-term (>3 hr) regulatory mechanisms do not appear to have a CyP450 component, but are TNF- and COX-2-dependent. A similar paradigm may apply to the ability of extracellular calcium to affect ion transport pathways in the mTAL.

inhibited AVP-mediated increases in cAMP in the mTAL, by a pertussis toxin-sensitive mechanism that was independent of AA metabolism (7). This finding is consistent with our studies showing that short-term regulation of some renal ion transport mechanisms is COX-independent and in some instances is mediated by cytochrome P450 metabolites such as 20-hydroxyeicosatetraenoic acid (2, 6, 8).

Hypercalcemia and loss of renal concentrating ability

Disturbances in renal concentrating ability, water intake, and loop of Henle function have been associated with hypercalcemia in humans and experimental animals (9, 10). In particular, inhibition of NaCl reabsorption by the TAL in hypercalcemia has been linked to increased prostaglandin levels in the kidney (11). Moreover, in a model of hypercalcemia induced by feeding rats dihydrotachysterol, levels of phospholipase A_2, and COX-2, but not COX-1, were elevated in the renal cortex, outer medulla, and inner medulla. Other studies have shown that experimentally-induced hypercalcemia was associated with increased prostanoid formation in the urine and increased renal COX-2 expression (12). The locus and mechanim(s) of this effect is not fully understood. Moreover, the linkage between COX-2 expression and PGE_2, formation in this model was not established (12). However, immunohistochemical data indicated an increase in COX-2 expression in the outer medulla, suggesting that COX-2 expression in the mTAL may increase after challenge with Ca_o^{2+}.

The total urinary Ca_o^{2+} concentration has been estimated to be 2.3+0.3 mM in a normal human ingesting a low calcium (10 mM) diet with unrestricted access to fluids (13). When a systemic Ca^{2+} load is excreted, small alterations in peritubular Ca^{2+} concentrations in the TAL attenuate its capacity for NaCl and Ca^{2+} reabsorption (14). However, urinary Ca^{2+} concentrations may increase substantially when water intake is restricted. Thus, during antidiuresis, AVP-induced osmotic water permeability in the terminal inner medullary collecting duct raises luminal Ca^{2+} concentrations to greater than 5 mM (14). The renal concentrating defect associated with hypercalcemia is characterized by resistance to arginine vasopressin (AVP), and an important role of the AT1 receptor for Ang II was observed in experimentally-induced hypercalcemia, associated with an increased expression of renal COX-2 (12). Localization and functional coupling of AT1A receptors in the mTAL was demonstrated by RT-PCR and measuring changes in Ca_i^{2+} by digital imaging microscopy (15). The Ang II-mediated increases in Ca_i^{2+} were completely abolished by the AT1 receptor antagonist, losartan. As Ang II increases mTAL TNF production (6), which increases COX-2 expression in this nephron segment (2), changes in Ca_i^{2+} may be part of a mechanism that promotes salt and water loss.

Ca_o^{2+}-mediated mechanisms that affect ion and water homeostasis

Ca_o^{2+}-sensing receptor (CaR), originally cloned from bovine parathyroid (16), also has been found in several nephron segments including the rat mTAL

(17). CaR are expressed in tissues that are involved in Ca^{2+} homeostasis including the parathyroid (16), parafollicular cells of the thyroid (18), and the kidney (19). Heterozygous and homozygous CaR knockout mice exhibit mild and severe alterations in Ca^{2+} homeostasis, respectively, confirming the importance of CaR in Ca^{2+} homeostasis (20). Moreover, the physiological relevance of this receptor in humans has been demonstrated by identifying hyper- and hypocalcemic disorders resulting from CaR mutations: familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism result from inactivating CaR mutations, while an autosomal dominant form of hypocalcemia is caused by activating mutations (21). Interestingly, persons with FHH have alterations in their handling of water, suggesting that CaR also may contribute to the regulation of water metabolism.

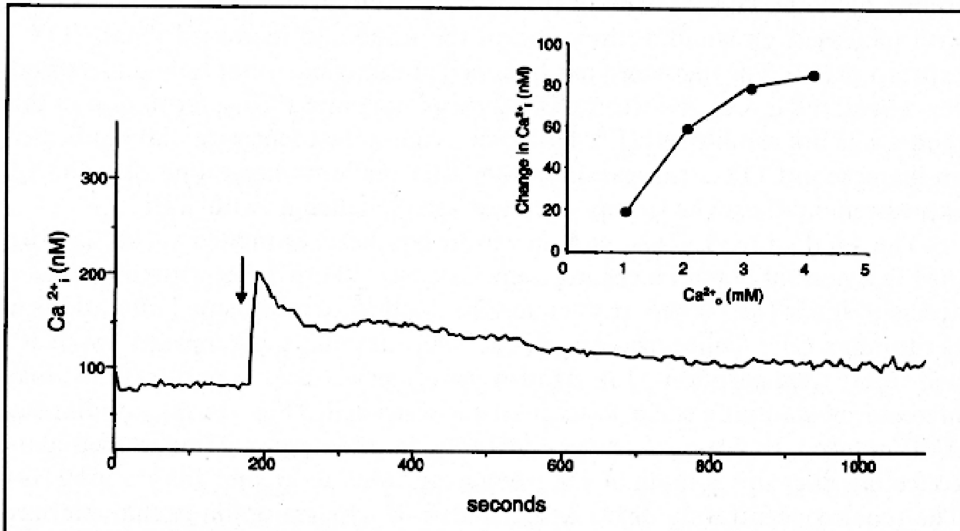

Fig. 2 Ca^{2+}_o increases Ca^{2+}_i in mTAL cells. Cells grown on glass coverslips, were preincubated for 30 min in Hank's Balanced Salt Solution containing 1.23 mM $CaCl_2$ then consecutively challenged with 1 mM $CaCl_2$ (insert; representative figure, n = 2) or 5 mM $CaCl_2$; (representative figure, n = 3). Ca^{2+}_i was determined using digital imaging microscopy in cells that were loaded with 2 μM Fura-2 acetoxymethyl ester for 30 min at 37°C/5% CO_2.

CaR are G-protein-coupled receptors that transduce extracellular Ca^{2+} binding into several intracellular signals including stimulation of inositol triphosphate (IP_3) production and release of Ca^{2+}_i. The release of Ca^{2+} from intracellular stores, in response to Ca^{2+}_o, implies that after stimulation of CaR, IP_3 and diacylglycerol release subsequent to activation of phosphatidylinositol-phospholipase will increase PKC activity. The effects of Ca^{2+}_o on changes in Ca^{2+}_i levels in mTAL cells were determined using digital imaging microscopy. Successive additions of 1 mM $CaCl_2$ increased Ca^{2+}_i in a dose-dependent manner and showed no desensitization (*Fig. 2*), as previously shown in rabbit and mouse cTAL (22, 23). Basal levels of $Ca^{2+}{}_i$ in cultured mTAL cells were approximately 100 nM and increased rapidly following additions of 5 mM Ca_o^{2+} (*Fig. 2*). The biphasic nature of the increase in Ca_i^{2+} may reflect an increase in Ca_i^{2+} by more than one mechanism. The rapid rise in Ca_i^{2+} is characteristic of release of Ca^{2+} from intracellular stores, while the sustained phase, in which the Ca_i^{2+} did not return to baseline levels as long as Ca_o^{2+} was present, may represent influx of Ca_o^{2+} *via* Ca^{2+} channels. We postulate that the rapid increase in Ca_i^{2+} after addition of Ca_o^{2+} is attributed to activation of the G-protein-coupled CaR, through which IP_3 production and release of Ca^{2+} is stimulated.

Ca_o^{2+} and poly-L-arginine increase PGE_2 production by mTAL cells

Calcium is a known activator of phospholipase A_2 (PLA_2), a major pathway by which AA is made available to various enzymes including COX, cytochrome P450 (CyP450), and lipoxygenases. Stimulation of CaR may, therefore, increase AA release for subsequent metabolism by COX-1 in mTAL cells. This mechanism is evident after short-term exposure (10 min) to Ca_i^{2+} or CaR agonists, as the effects of high (5 mM) Ca_i^{2+} and Gd^{3+} were abolished after inhibition of PLA_2 activity (24) or addition of 17-octadecynoic acid, a specific inhibitor of CyP450 monooxygenase. However, recent work from our laboratory has demonstrated that different eicosanoid mediators regulate the short- and long-term effects of Ang II (6). Moreover, the coupling of CaR to intracellular signaling pathways may vary according to the magnitude of the stimulus and the parameter that is assessed (25). Thus, mTAL cells were challenged for various times with 1.7 or 2.0 mM Ca_o^{2+} and PGE_2 levels were determined by ELISA. Control levels of PGE_2 increased slightly over the time course studied, as did Ca_o^{2+}-stimulated PGE_2 formation after a 1 hr challenge (*Fig. 3 A*). In contrast, substantial increases in PGE_2 production were observed when cells were challenged with either 1.7 or 2.0 mM Ca_o^{2+} for 3 or 9 hr (*Fig. 3 A*). The magnitude of the responses at 1 hr vs. 3 and 9 hr may reflect differences in the mechanisms by which PGE_2 was produced (i.e.; a COX-1-dependent mechanism at 1 hr and a COX-2-dependent mechanism at 3 and 9 hr). Control experiments in which extracellular Cl^- concentrations were increased, (by adding NaCl), without changing Ca_o^{2+} had no effect on PGE_2 production (data not shown). Cells challenged for 9 hr with the CaR agonist, poly-L-arginine, also produced significantly more PGE_2 than control cells (*Fig. 3 B*). As PMA, a direct activator of PKC, also increases COX-2 expression in mTAL cells (2), it is possible that activation of PKC may be one mechanism by which COX-2 expression increases after challenge with Ca_o^{2+} and CaR agonists.

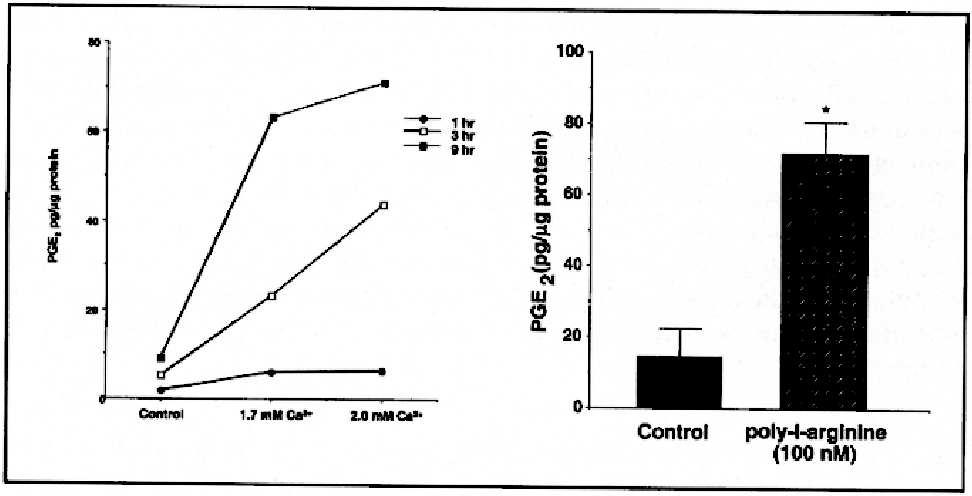

Fig. 3. Effects of Ca_o^{2+} and poly-l-arginine on PGE_2 formation by mTAL cells. Primary cultures of mTAL cells were quiesced in RPMI 1640 containing 0.42 mM $CaCl_2$ and 0.5% FCS for 18 hr then exposed for (A) various times to Ca_o^{2+}, (representative experiment, n = 2) or (B) 9 hr to poly-L-arginine; (*): $p < 0.01$, n = 3. PGE_2 was determined by ELISA. "Control" indicates that cells were incubated in media containing 0.42 mM Ca^{2+}; this amount should be added to the amounts used to challenge the cells to obtain total Ca_o^{2+} present.

Ca_o^{2+}-mediated mechanisms that affect ion and water homeostasis

Stimulation of CaR by Ca_o^{2+} was recently shown to acutely increase production of 20-HETE, which inhibits activity of the apical 70 pS K^+ channel in the mTAL (26). This channel is important for K^+ recycling and maintaining activity of the apical Na^+-K^+-2 Cl^- cotransporter, a major determinant of salt and water regulation by the mTAL (27). PGE_2 also inhibits the Na^+-K^+-2Cl^- cotransporter, as well as the Na^+ pump and apical K^+ channel (28—30). However, the short-term effect of Ca_o^{2+} on the apical K^+ channel activity occurred by a cyclooxygenase-independent mechanism. Previous work from our laboratory has shown that a temporal dependency determines the involvement of either cytochrome P450- or COX-derived AA metabolites on mTAL ion transport mechanisms. For instance, the short-term (15 min) effects of Ang II were mediated *via* a cytochrome P450-dependent mechanism, while long-term effects (>3—4 hr) were indomethacin-sensitive and involved a mechanism that also was dependent on the expression of TNF production (6) (*Fig. 1*). It is possible that regulation of ion transport mechanisms mediated by AA metabolites in the mTAL have different characteristics that are determined by the duration of the stimulus. Thus, while the short-term effects of Ca_o^{2+} are mediated by 20-HETE and appear to involve activation of the CaR, we propose that the ability of Ca_o^{2+} and CaR agonists to increase PGE_2

production include a TNF-dependent mechanism that facilitates COX-2 expression.

Our previous finding that Ang II increases TNF production in the mTAL suggests that, under certain circumstances, this cytokine is critical to the activity of Ang II on ion transport function in this nephron segment (6). As these effects were attributed to an increase in COX-2 mRNA accumulation, protein expression and activity, induction of TNF may be essential to the regulation of COX-2 expression in the mTAL. Thus, other molecules that affect TNF production by these cells also may influence transport mechanisms *via* prostanoids derived from COX-2. The functional implications of such a mechanism are those predicted by increased levels of PGE_2; i.e. inhibition of the Na^+ pump, cotransporter, and apical 70 pS K^+ channel are all mechanisms that promote salt and water excretion. We hypothesize that calcium, *via* stimulation of the CaR, may be a regulator of TNF production and COX-2 expression in the mTAL. Such an interaction may provide a mechanism by which eicosanoids derived from distinct biochemical pathways (CyP450 vs. COX) and subject to different regulatory influences might reinforce each other's actions. For instance, while calcium rapidly increases 20-HETE formation by the mTAL *via* a putative CaR-dependent mechanism, stimulation of PGE_2 synthesis *via* a COX-2-dependent mechanism would require considerably more time. The net effect of these temporally dissociated events would be the synthesis of two different eicosanoids, 20-HETE and PGE_2, with similar effects on mTAL ion transport pathways. The role of COX-2 in this setting also may include an important mechanism for the conversion of 20-HETE to prostanoid-like compounds.

Recently, compounds termed "calcimimetics", which mimic or potentiate the effects of Ca_o^{2+} at the level of the CaR have been discovered (31). These agents were designed as a means of providing pharmacological manipulation of plasma levels of parathyroid hormone (PTH), and the first-generation calcimimetic, NPS R-568, has exhibited promising results in clinical trials for primary hyperthyroidism and hyperparathyroidism secondary to chronic renal insufficiency (32). Our studies suggest that these molecules also may offer a novel means of affecting salt and water reabsorption. This mechanism may provide the rationale for the use of these molecules in conditions, such as hypertension and congestive heart failure, in which manipulation of extracellular fluid volume has a beneficial effect.

REFERENCES

1. Yang T, Singh I, Pham H, Sun D, Smart A, Schnermann JB, Briggs JP. Regulation of cyclooxygenase expression in the kidney by dietary salt intake. *Am J Physiol* 1998; 274: F481-F489.

2. Ferreri NR, An S-J, McGiff JC. Cyclooxygenase-2 expression and function in the medullary thick ascending limb. *Am J Physiol* 1999; 277: F360-F368.
3. Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, Breyer MD. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Invest* 1994; 94: 2504—2510.
4. Vio CP, An S, Cespedes C, McGiff JC, Ferreri NR. Adrenalectomy induces recruitment of thick ascending limb cells containing cyclooxygenase-2. (*submitted for publication*).
5. Stanton BA. Regulation by adrenal corticosteroids of sodium and potassium transport in loop of Henle and distal tubule of rat kidney. *J Clin Invest* 1986; 78: 1612—1620.
6. Ferreri NR, Escalante BA, Zhao Y, An S, McGiff JC. Angiotensin II induces TNF production by the thick ascending limb: functional implications. *Am J Physiol* 1998; 274: F148-F155.
7. Firsov D, Aarab L, Mandon B, Siaume-Perez S, Rouffignac CD, Charbardes D. Arachidonic acid inhibits hormone-stimulated cAMP accumulation in the medullary thick ascending limb of rat kidney by a mechanism sensitive to pertussis toxin. *Pflugers Arch* 1995; 429: 636—646.
8. Escalante BA, Ferreri NR, Dunn CE, McGiff JC. Cytokines affect ion transport in primary cultured thick ascending limb of Henle's loop cells. *Am J Physiol* 1994; 266: C1568-C1576.
9. Gill JR, Bartter FC. On the impairment of renal concentrating ability in prolonged hypercalcemia and hypercalcuria in man. *J Clin Invest* 1961; 40: 716—722.
10. Peterson LN. Vitamin D-induced chronic hypercalcemia inhibits thick ascending limb NaCl reabsorption in vivo. *Am J Physiol* 1990; 259: F122—F129.
11. Peterson LN, McKay AJ, Borzecki JS. Endogenous prostaglandin E_2 mediates inhibition of rat thick ascending limb Cl reabsorption in chronic hypercalcemia. *J Clin Invest* 1993; 91: 2300—2407.
12. Mangat H, Peterson LN, Burns KD. Hypercalcemia stimulates expression of intrarenal phospholipase A_2 and prostaglandin H synthase-2 in rats. *J Clin Invest* 1997; 100: 1941—1950.
13. Jacobson A, Singhal P, Mandin H, Hyne J. Urinary ionic calcium and binding sites in normocalciuric idiopahtic calcium urolithiasis. *Invest Urol* 1979; 17: 218—231.
14. Sands JM, Naruse M, Baum M, Jo I, Hebert SC, Brown EM, Harris HW. Apical extracellular calcium/polyvalent cation-sensing receptor regulates vasopressin-elicited water permeability in rat kidney inner medullary collecting duct. *J Clin Invest* 1997; 99: 1399—1405.
15. Bouby N, Hus-Citharel A, Marchetti J, Bankir L, Corvol P, Llorens-Cortes C. Expression of type 1 angiotensin II receptor subtypes and angiotensin II-induced calcium mobilization along the rat nephron. *J Am Soc Nephrol* 1997; 8: 1658—1667.
16. Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC. Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature* 1993; 366: 575—580.
17. Riccardi D, Lee WS, Lee K, Segre GV, Brown EM, Hebert SC. Localization of the extracellular Ca^{2+} sensing receptor and PTH/PTHrP receptor in rat kidney. *Am J Physiol* 1996; 271: F951—F956.
18. Freichel M, Zink-Lorenz A, Holloschi A, Hafner M, Flockerzi V, Raue F. Expression of a calcium-sensing receptor in a human medullary thyroid carcinoma cell line and its contribution to calcitonin secretion. *Endocrinology* 1996; 137: 3842—3848.
19. Riccardi D, Park J, Lee W, Gamba G, Brown EM, Hebert SC. Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation/sensing receptor. *Proc Natl Acad Sci USA* 1995; 92: 131—135.
20. Ho C, Connor DA, Pollak MR, Ladd DJ, Kifor O, Warren HR, Brown EM, Seidman JG, Seidman CE. A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Nature Genet* 1995; 11: 389—394.
21. Hebert SC, Brown EM. The scent of an ion: calcium-sensing and its roles in health and disease. *Curr Opin Nephrol Hypertens* 1996; 5: 45—53.

22. Desfleurs E, Wittner M, Pajaud S, Nitschke R, Rajerison RM, DiStefano A. The Ca^{2+}-sensing receptor in the rabbit cortical thick ascending limb (cTAL) is functionally not coupled to phospholipase C. *Pflugers Arch-Eur J Physiol* 1999; 437: 716—723.
23. Paulais M, Baudouin-Legros M, Teulon J. Functional evidence for a Ca^{2+}/polyvalent cation sensor in the mouse thick ascending limb. *Am J Physiol* 1996; 271: F1052—F1060.
24. Wang W-H, Lu M, Balazy M, Hebert SC. Phospholipase A_2 is involved in mediating the effect of extracelluar Ca^{2+} on apical K^+ channels in rat TAL. *Am J Physiol* 1997; 273: F421—F429.
25. McGehee DS, Aldersberg M, Liu K-P, Hsuing S-C, Heath MJS, Tamir H. Mechanism of extracellular Ca^{2+} receptor-stimulated hormone release from sheep thyroid parafollicular cells. *J Physiol* 1997; 502: 31—44.
26. Wang W-H, Lu M, Hebert SC. Cytochrome P-450 metabolites mediate extracellular Ca^{2+}-induced inhibition of apical K^+ channels in the TAL. *Am J Physiol* 1996; 271: C103-C111.
27. Wang W-H, Hebert SC, Giebisch G. Renal K^+ channels: structure and function. *Annual Rev of Physiol* 1997; 59: 413—436.
28. Kaji M, Chase JrHS, Eng JP, Diaz J. Prostaglandin E_2 inhibits Na-K—2Cl cotransport in medullary thick ascending limb cells. *Am J Physiol* 1996; 271: C354—C361.
29. Wald H, Scherzer P, Rubinger D, Popovtzer MM. Effect of indomethacin in vivo and PGE_2 in vitro on mTAL Na-K-ATPase of the rat kidney. *Pflugers Arch* 1990; 415: 648—650.
30. Liu HS, Ferreri NR, Nasjletti A, Wang W-H. Vasopressin and PGE_2 regulate the acativity of the apical 70 pS K^+ channel in the thick ascending limb of the rat kidney. *Am J Physiol* 2000; 278: C905—C913.
31. Nemeth EF, Steffey ME, Hammerland LG, Hung BC, VanWagenen BC, DelMar EG, Balandrin MF. Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proc Natl Acad Sci USA* 1998; 954040—4045.
32. Wada M, Nagano N, Nemeth EF. The calcium receptor and calcimimetics. *Curr Opin Nephrol Hypertens* 1999; 8: 429—433.

Received: October 3, 2000
Accepted: October 28, 2000

Author's address: Dr. Nicholas R. Ferreri, Dept of Pharmacology, New York Medical College, Valhalla, USA.
E-mail: nick_ferreri@nymc.edu