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Cell cycle-dependent and kinase-specific regulation of the apical Na/H exchanger and the Na,K-ATPase in the kidney cell line LLC-PK$_1$ by calcitonin

(sodium reabsorption/sodium pump)

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ABSTRACT **Calcitonin (CT), which regulates serum calcium through its actions in bone and the kidney tubule, also has a potent natriuretic effect *in vivo*. Na reabsorption in the proximal kidney tubule is mostly dependent on the activity of the Na,K-ATPase and the apical Na/H exchanger. We have previously shown that CT regulates the activity of the Na,K-ATPase in the proximal kidney tubule cell line LLC-PK$_1$ in a cell cycle-dependent manner. We report here that, in the same cells, CT also regulates the Na/H exchanger through a cell cycle-specific activation of the Ca/calmodulin-dependent protein kinase II. In G$_2$ phase, no changes in ethylisopropyl amiloride-sensitive ^{22}Na uptake is observed, despite an increase in cAMP. In contrast, the hormone inhibits the apical exchanger when the cells are in S phase, resulting in an 80% inhibition of ^{22}Na uptake. These results demonstrate that CT affects the activity of the two major proximal tubule Na transport systems and may help clarify the mechanisms by which CT regulates Na$^+$ reabsorption.**

Calcitonin (CT), a 32-amino acid hormone with profound hypocalcemic effects due to its action in bone and the kidney (1, 2), also increases urinary excretion of Na (3). The mechanisms underlying this effect of CT on the kidney tubule, however, have not been elucidated. In the kidney, Na reabsorption results mostly from activity of the basolateral Na,K-ATPase and the apical Na/H exchanger (NHE) in the proximal tubule and/or Na channels in the distal tubule (4, 5). We have reported (6) that CT regulates Na,K-ATPase in a proximal kidney tubule cell line (LLC-PK$_1$) (7), with the effect of the hormone depending on the position of the cells in the cell cycle; during G$_2$, CT increases cAMP via the stimulatory guanine nucleotide-binding protein (G$_s$ protein), leading to a 2-fold increase in ouabain binding; during S phase, this pathway is blocked by a pertussis-sensitive inhibitory G protein (G$_i$ protein), and CT induces activation of protein kinase C (PKC) and a 4-fold decrease in ouabain binding.

These results demonstrated a link between CT and Na pumps but did not indicate whether the NHE was also affected. The NHEs are a family of electroneutral antiporters present in the plasma membranes of most mammalian cells (8, 9). They fulfill multiple cellular functions including the regulation of cell volume and intracellular pH (pH$_i$) and are involved in the transepithelial transport of Na$^+$ in the proximal tubule. Three NHE isoforms have been reported in the literature. NHE1 is ubiquitous, present on the basolateral domain of epithelial cells and on the plasma membrane of all cells. It is highly sensitive to amiloride and is thought to function primarily in the regulation of pH$_i$ and in signal transduction (8–10). The second NHE isoform is found in the apical membrane domain of renal, intestinal, and gall bladder epithelia (10). This apical NHE is less sensitive to inhibition by amiloride and is primarily involved in Na$^+$ reabsorption and proton secretion. A third isoform has recently been reported in the intestine (11) but it is not yet well characterized.

CT activates several signal transduction pathways (6, 12–14), some differentially along the kidney tubule (15, 16), which could affect the activity of the NHEs (17). The major objective of this study was therefore to determine whether CT regulates the NHEs in LLC-PK$_1$ cells, whether such effects varied with the phase of the cell cycle, and how the observed responses, if any, could be related to the changes in Na,K-ATPase activity and the natriuretic effect of the hormone.

MATERIALS AND METHODS

Materials. Salmon calcitonin was from Rorer Central Research (King of Prussia, PA). Ethylisopropyl amiloride (EIPA) was obtained from Merck Sharp and Dohme. Ouabain and all other chemicals were from Sigma. [^{3}H]Ouabain (specific activity, 20 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]thymidine (specific activity, 15 Ci/mmol), and ^{22}NaCl (specific activity, 100–1000 mCi per mg of sodium) were from Amersham.

Cells. LLC-PK$_1$ (clone 4) cells and PKE6, a mutant derived from clone 4, were obtained from Carolyn Slayman (Yale University); both cell lines were maintained in α-MEM containing 10% fetal bovine serum at 37°C with 5% CO$_2$/95% air. Synchronization of cells and the [^{3}H]ouabain binding assay were carried out as described (6). Experiments were performed at the first G$_2$ phase (14 hr after removal of 5-fluoro-2′-deoxyuridine) and the second S phase (18 hr), as previously determined (6). For the studies on PKE6 cells, the phases of the cell cycle after removal of fluorodeoxyuridine were characterized as described (6) and the times of G$_2$ phase (13 hr) and S phase (21.5 hr) were used in all experiments.

Assay of ^{22}Na$^+$ Uptake. EIPA-sensitive ^{22}Na$^+$ uptake was measured as described (18). The standard assay was performed by aspirating the medium from the culture dishes, adding 2.0 ml of Na$^+$-free Earle's solution [140 mM choline chloride/5.4 mM KCl/1.8 mM CaCl$_2$/0.8 mM MgSO$_4$/5 mM glucose/25 mM Hepes adjusted to pH 7.4 with Tris(hydroxymethyl)amino methane] containing 50 mM NH$_4$Cl and incubating the cells for 30 min with or without CT. This medium was then replaced with NH$_4^+$-free Earle's solution containing 15 mM NaCl, ^{22}Na$^+$ at a final specific activity of

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Abbreviations: CT, calcitonin; NHE, Na/H exchanger; G protein, guanine nucleotide binding protein; PKC, protein kinase C; EIPA, ethylisopropyl amiloride; CaM kinase II, Ca/calmodulin-dependent kinase II; CaMK IP, CaM kinase inhibitory peptide.

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1 μCi/ml, and 0.5 mM ouabain, with or without EIPA (100 μM) for 2 min. At the end of the uptake period, the plates were rapidly rinsed with six changes of ice-cold 0.1 M $MgCl_2$ and air dried, and the cells were solubilized in 10% trichloroacetic acid for counting in parallel dishes.

Ca/Calmodulin-Dependent Protein Kinase II (CaM Kinase II) Peptide Inhibitors. Inhibitory peptides for CaM kinase (CaMK IP) were as follows. CaMK IP2 is a synthetic peptide corresponding to amino acids 281–302 of the α subunit of rat brain CaM kinase II. CaMK IP1 is identical except for an alanine substituted for threonine at position 286, a substitution that improved the inhibition of CaM kinase II-dependent phosphorylation of synapsin I (19). This peptide inhibits the catalytic activity of rat brain CaM kinase II with a K_i of 5 μM when assayed with synapsin I as substrate, and it has been previously used to inhibit CaM kinase II activity in intact nerve terminals (20). The other inhibitor, CaMK IP2, was 2- to 3-fold less effective than CaMK IP1 (20).

Assay of CaM Kinase II. CaM kinase II activity in the total cell lysates was assayed according to Jefferson *et al.* (21) with the following modifications. A synthetic peptide corresponding to amino acids 281–291 of the α subunit of CaM kinase II was used in our assay as described (21, 22). The reaction was terminated by rapidly spotting the reaction mixtures on Whatman P81 phosphocellulose filter papers, which were immediately placed in a large vol (300 ml) of 75 mM phosphoric acid solution and washed. The filter papers were air dried and incorporation of ^{32}P into substrate was quantitated by counting Cerenkov radiation. The CaM kinase II specific activity was calculated by subtracting the kinase activity measured in the presence of the protein kinase II inhibitor CaMK IP1 (50 μM) from the total activity and was expressed as pmol per min per mg of protein.

Other Methods. Permeabilization of the cells was carried out with digitonin as described (6). Na,K-ATPase was assayed according to Aperia *et al.* (23). Immunoblot analysis with anti-CaM kinase II antibody G-301, raised in rabbit against a synthetic peptide corresponding to residues 281–302 of the α subunit of rat brain CaM kinase II, was carried out according to Yamagata *et al.* (22).

Statistics. When applicable, all data in a series of experiments were compared by performing first a one-way ANOVA followed, when significant, by comparisons between experimental groups using the Scheffe *F* test. All differences that reached a probability of 5% or lower were considered statistically significant.

RESULTS

To investigate whether CT affected NHE activity, we first tested the effects of CT on the EIPA-sensitive uptake of ^{22}Na by acid-loaded LLC-PK_1 cells. As shown in Fig. 1, the hormone dose dependently inhibited up to 80% of the EIPA-sensitive ^{22}Na uptake in S phase ($P < 0.001$), whereas at G_2 phase, despite an increase in cAMP, CT had no effect on Na uptake. Hence, in S phase, CT mimics the effects of amiloride on the NHE. Conversely (Fig. 2), amiloride and EIPA could mimic the effects of CT on [^3H]ouabain binding in S phase of the cell cycle, inducing a 70–80% inhibition ($P < 0.001$). In contrast, inhibition of the activity of the NHE during G_2 phase had no detectable effect on the Na,K-ATPase. When tested at a concentration (10 μM) that affects only the Na channels (24), amiloride did not affect ouabain binding in LLC-PK_1 cells whether the cells were in G_2 or S phase.

Thus, in S phase of the cell cycle, CT mimicked the effects of amiloride on Na uptake and amiloride mimicked the effects of CT on ouabain binding, thereby strongly suggesting that the NHE may be an effector for CT in these cells. In contrast, the G_2 phase cAMP-mediated activation of the Na,K-ATPase

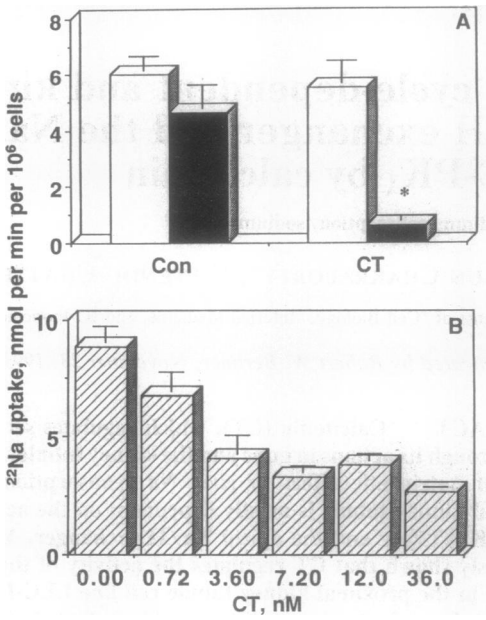

FIG. 1. Effects of CT on EIPA-sensitive ^{22}Na uptake by acid-loaded LLC-PK_1 (clone 4) cells at G_2 (open bars) and S phase (solid bars). $^{22}Na^+$ uptake was measured at G_2 and S phase. (*A*) CT inhibits ^{22}Na uptake by 70–80% ($P < 0.001$) in S phase; no effects are detected in G_2 phase. (*B*) Inhibition of ^{22}Na uptake by CT in S phase is dose dependent. Con, control. *, $P < 0.001$.

(6) apparently does not involve changes in the activity of the NHEs.

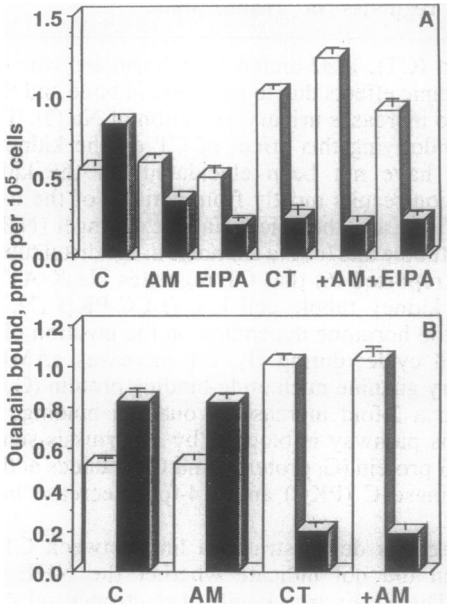

FIG. 2. Effects of CT, EIPA, and amiloride on [^3H]ouabain binding in LLC-PK_1 (clone 4) cells at G_2 and S phase. Monolayer of synchronized cells (70–75% confluency) at G_2 and S phase of their cell cycle were preincubated for 15 min at 37°C in Hanks' balanced salt solution (HBSS) followed by incubation with or without salmon CT (12 nM), amiloride (10 or 75 μM), or EIPA (10 μM) for 30 min under the same conditions. [^3H]Ouabain binding was measured and results are expressed as pmol of ouabain bound per 10^5 cells. +AM, CT + amiloride; +EIPA, CT + EIPA. CT significantly stimulated [^3H]ouabain binding in G_2 phase (open bars) and inhibited it in S phase (solid bars). Amiloride (75 μM) and EIPA (10 μM), two blockers of the NHEs, inhibited [^3H]ouabain binding in S phase, an effect similar to that of CT, but they had no effect at G_2 phase (*A*). When used at 10 μM, which inhibits the Na channels but not the NHE, amiloride had no effect in either S or G_2 phase (*B*).

We then determined which NHE was responsible for the changes observed in S phase. Although two different isoforms of the NHE are expressed on different membrane domains in fully confluent and polarized LLC-PK$_1$ cells cultured on a filter (20), this system could not be used for studies related to the cell cycle, cell proliferation being contact inhibited. Consequently, and as described before (6), we have used cells at 70–80% confluence—i.e., in transition between rapid growth and confluence.

First, we observed that rapidly growing LLC-PK$_1$ cells, which express only the basolateral isoform (20), do not respond to CT with changes in ouabain binding or Na^+ uptake (data not shown). Second, we titrated the effects of EIPA on ouabain binding in cells at S phase (Fig. 3) and found that when EIPA was used at a concentration of 40 nM, which corresponds to the IC_{50} for the basolateral NHE, it was unable to affect ouabain binding, whereas 10 μM EIPA, which blocks the apical NHE, could mimic the effects of CT. To verify that it is indeed the apical isoform that is affected by CT, we also used a mutant cell line (PKE6) [obtained from the LLC-PK$_1$ cell line as previously described for clone PKE5 (25)] that is deficient in expression of the basolateral form of the exchanger. Despite a 2.7-fold decrease in expression of the basolateral exchanger over the parental cell line and a 4-fold decrease in the ratio of the two exchangers (as determined by EIPA titration), we observed that the effects of CT were of a similar nature and amplitude in PKE6 cells in all the assays (data not shown).

Taken together, the results of EIPA titration on ouabain binding, the lack of effects of CT on rapidly growing clone 4 cells (which express only the basolateral NHE), and the ability of CT to induce identical effects in partially confluent clone 4 and PKE6 cells suggest that the decrease in ^{22}Na uptake in response to CT is due to inhibition of the apical NHE.

Our previous studies having shown that the CT receptor is coupled to different signal transduction pathways during the cell cycle, we then determined which second messengers were responsible for inhibition of the apical NHE. Since binding of CT in S phase activates the PKC pathway (6), we first determined the role played by this kinase. Surprisingly, and in contrast with ouabain binding, inhibition of PKC by sphingosine or its activation by phorbol esters (phorbol dibutyrate) did not prevent or mimic, respectively, the effects of CT on Na uptake (Fig. 4*A*).

Thus, neither the CT-induced increase in cAMP in G_2 phase nor the CT-induced or phorbol ester-mediated activation of PKC in S phase could inhibit EIPA-sensitive ^{22}Na uptake. Because CT also induces an increase in intracellular

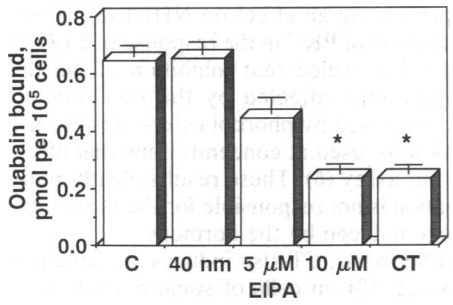

FIG. 3. Dose-dependent inhibition of [^{3}H]ouabain binding to LLC-PK$_1$ (clone 4) cells in S phase by EIPA. LLC-PK$_1$ (clone 4) cells were synchronized and [^{3}H]ouabain binding was measured at S phase. Although EIPA dose dependently inhibits [^{3}H]ouabain binding, micromolar concentrations are necessary to mimic the effects of CT (maximum inhibition, 64% ± 5%; $P < 0.01$; 10 μM EIPA), thereby suggesting inhibition of the apical exchanger. C, control.

Ca (12, 15), we then determined whether activation of the CaM kinase II was involved in inhibiting the apical NHE.

For this purpose, we tested the effects of two synthetic inhibitory peptides (CaMK IP1 and -2) on the responses elicited by CT. These peptides are analogs of the CaM kinase II binding domain and thereby compete with CaM kinase II for binding on its substrates (20). We first determined the ability of CT to activate CaM kinase II in these cells by assaying total kinase activity in cells treated with or without CT at S phase. The total Ca-stimulated (activity observed after addition of Ca and calmodulin) kinase activity (i.e., CaMK IP1 inhibitable) did not change significantly after CT. In contrast, the autonomous CaM kinase II activity (activity observed in the cells without addition of Ca and calmodulin during the assay) was increased 5-fold after treatment with CT (Fig. 4*B*). Thus, CT induces an increase in CaM kinase II activity, with no change in the amount of the enzyme (data not shown). When introduced into reversibly permeabilized cells, CaMK IP1 and -2 had no effects by themselves but prevented >80% of the inhibitory effect of CT on EIPA-sensitive ^{22}Na uptake ($P < 0.01$) (Fig. 4*C*), suggesting that activation of CaM kinase II was required for CT to exert its full effect on the apical NHE. We concluded from these experiments that CT-induced inhibition of the apical NHE during S phase occurs predominantly via activation of the CaM kinase II.

Finally, to determine the contribution of the inhibition of the apical NHE to the CT-induced reduction in Na,K-ATPase activity, we measured the effects of CaM kinase II inhibition on ouabain binding in response to CT in S phase. The changes in ouabain binding in response to CT were of a significantly lesser amplitude in cells treated with the inhibitory peptide CaMK IP2 (50% instead of 80% inhibition in LLC-PK$_1$ cells; $P < 0.01$). However, and in contrast with its effects on ^{22}Na uptake, inhibition of CaM kinase II failed to completely abolish the effects of CT on the Na,K-ATPase (Fig. 4*D*). The fact that the Na,K-ATPase could be inhibited by CT independently from NHE-mediated changes in intracellular Na^+ was confirmed by measuring both [^{3}H]ouabain binding and Na,K-ATPase activity in LLC-PK$_1$ (clone 4) cells in which intracellular Na^+ was clamped at 150 mM by addition of nystatin (0.1 unit/μl) to the incubation buffer. CT could still inhibit up to 60% of the binding and enzyme activity (instead of up to 80%) in the presence of the Na^+ ionophore (data not shown). Thus, inhibition of the Na,K-ATPase by CT in S phase is only partially due to NHE-mediated changes in intracellular Na^+.

Regulation of the activity of the Na pump by CT is therefore mediated by two independent intracellular signaling events: activation of PKC (6), which may act directly on the Na,K-ATPase (26, 27), and activation of the CaM kinase II, which acts indirectly by inhibiting the apical isoform of the NHE and probably lowers intracellular Na^+.

DISCUSSION

The main observations reported in the present study are that (*i*) the apical NHE is a major effector of CT in the proximal kidney tubule cell line LLC-PK$_1$ (clone 4). (*ii*) Binding of CT to its receptor activates in a cell cycle-dependent manner not only the cAMP and PKC pathways (6) but also CaM kinase II, which leads to a profound inhibition of the apical NHE. (*iii*) The Na pump is independently inhibited via the PKC pathway. These results demonstrate a direct link between CT and Na transport in a proximal kidney tubule cell line, a response possibly related to the potent natriuretic effect of the hormone *in vivo* (2, 3).

These results also confirm our previous observation that the CT receptor, recently identified as a member of a subfamily within the G-protein-coupled receptors (28), is cou-

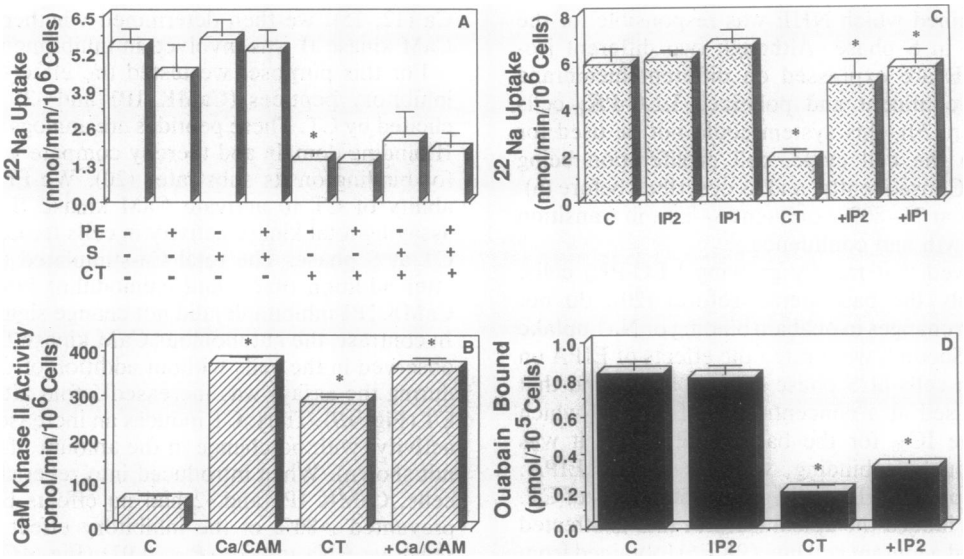

FIG. 4. Role of CaM kinase II in mediating the effects of CT on the NHE. (*A*) Effects of stimulation and inhibition of PKC on EIPA-sensitive ^{22}Na uptake by acid-loaded LLC-PK$_1$ (clone 4) cells in S phase. LLC-PK$_1$ cells were acid loaded in the presence or absence of phorbol dibutyrate (PdBU) (PE) (1 μM) or sphingosine (S) (10 μM) and ^{22}Na uptake was measured. Neither activation of PKC by PdBU nor its inhibition by sphingosine affected ^{22}Na uptake or its response to CT. (*B*) Effect of CT on CaM kinase II kinase activity in LLC-PK$_1$ (clone 4) 6 cells in S phase. LLC-PK$_1$ (clone 4) cells in S phase were treated with or without CT (12 nM) for 30 min in HBSS and washed; CaM kinase II in the total cell lysates was assayed according to Jefferson *et al.* (21) and normalized as described. CT treatment induced a significant increase in the CaM kinase II activity, to ≈80% of maximal stimulation of the enzyme by exogenous Ca^{2+} (1.5 mM) and calmodulin (3 μg per 50 μl of incubation mixture). (*C*) Effects of inhibition of CaM kinase II by synthetic peptides IP1 and IP2 on EIPA-sensitive ^{22}Na uptake by acid-loaded LLC-PK$_1$ (clone 4) cells in S phase. LLC-PK$_1$ (clone 4) cells were permeabilized for 6 min by addition of 20 μM digitonin in the presence or absence of CaMK IP2 (150 μM) or CaMK IP1 (50 μM). Cells were washed twice in HBSS (pH 7.4) and acid loaded in the presence or absence of CT (12 nM); ^{22}Na uptake was measured. CaMK IP1 and -2 had no effect by themselves on ^{22}Na uptake by LLC-PK$_1$ cells, but both inhibitors blocked 79% ($P < 0.01$) and 83% ($P < 0.01$) of the inhibitory effect of CT on ^{22}Na uptake. (*D*) Effects of inhibition of CaM kinase II by CaMK IP2 on [^{3}H]ouabain binding in LLC-PK$_1$ cells in S phase. LLC-PK$_1$ (clone 4) cells were loaded in S phase with CaMK IP2 (50 μM) by transient permeabilization of the cells with digitonin in the presence or absence of CT (12 nM), and [^{3}H]ouabain binding was assayed. There was significant [$P < 0.01$; CT vs. control (C)] inhibition of ouabain binding by CT, and inhibition of CaM kinase II by CaMK IP2 only partially blocked (20–30%; $P < 0.01$ vs. CT-treated cells and $P < 0.01$ vs. control) the inhibitory effects of CT on the Na,K-ATPase in S phase.

pled to different signal-transduction pathways in a cell cycle-dependent manner (6).

Our conclusion that some of the effects of CT are mediated via alteration of the activity of the NHEs is supported by several observations. Of particular importance are the findings that EIPA-sensitive ^{22}Na uptake is profoundly inhibited by the hormone, demonstrating an EIPA-like effect of CT, and that amiloride and EIPA have CT-like effects on ouabain binding. Although Na channels are also inhibited by amiloride and its derivatives, these inhibitors, when used at concentrations that affect Na channels but not the NHEs (24), did not mimic the effects of CT on ouabain binding. These results imply that these channels, whose importance in Na reabsorption is most prominent in the distal tubule (4, 5), are not involved in the response of this proximal tubule cell line to CT. CT may also regulate Na/K/Cl cotransport in a kidney thick ascending limb cell line (29), but it is unlikely that this cotransporter would be involved in the changes we report here since it is not sensitive to amiloride or EIPA, whereas it is the EIPA-sensitive component of Na uptake that is inhibited by CT. Hence, the observed effects of CT on Na uptake are most likely due to inhibition of the NHE(s) and not to effects on Na channels or the Na/K/Cl cotransporter.

Since NHEs are encoded by a gene family of which only the apical exchanger is involved in Na reabsorption (4), we determined which NHE isoform was affected by CT in LLC-PK$_1$ cells. Three independent sets of experiments indicate that it is inhibition of the apical NHE that is responsible for the decrease in EIPA-sensitive ^{22}Na uptake after CT treatment. First, inhibition of the basolateral NHE with low concentrations of EIPA failed to mimic the changes in ouabain binding induced by CT. Second, rapidly growing cells, which express only the basolateral exchanger (30), failed to respond to CT with changes in ^{22}Na uptake. Third, the mutant cell line PKE6 responded to CT as well as the parental clone 4 cell line despite an ≈3-fold decrease in the level of expression of the basolateral exchanger. It therefore seems warranted to conclude that CT inhibits specifically the apical isoform of the NHE, a finding that makes the effect of this hormone more physiologically relevant.

These changes in Na uptake were exclusively observed in cells in S phase and not during G_2 phase of the cell cycle. We consequently expected this inhibition of the apical NHE to be mediated by PKC, based on our previous observation that this kinase is activated during S phase (6). On the other hand, most of the reports in the literature indicate that PKC activates, rather than inhibits, Na/H exchange and this, for the most part, is via an effect on NHE1 (8). Further examination of the role of PKC in the response of LLC-PK$_1$ (clone 4) cells to CT revealed that inhibition of Na uptake was neither significantly affected by the PKC inhibitor sphingosine nor mimicked by phorbol esters, despite the fact that both agents were used at concentrations that affect ouabain binding in our assay (6). These results clearly indicated that PKC activation is not responsible for the profound inhibition of Na uptake induced by the hormone.

On the other hand, CT also induces an elevation of Ca_i in osteoclasts (12, 13), in cells of some portion of the kidney tubule (15), and in cells overexpressing the CT receptor (31), and activation of CaM kinase(s) has been reported to inhibit Na/H exchange (19, 32, 33). We found not only that CT induces a marked increase in CaM kinase II activity, but also that the effects of CT on EIPA-sensitive ^{22}Na uptake were blocked by inhibiting CaM kinase II. We therefore concluded that activation of CaM kinase II is responsible for CT-

induced inhibition of the apical isoform of the NHE in these cells.

The CaMK IP, however, failed to block the effects of CT on ouabain binding, under conditions where the PKC inhibitor sphingosine did (6), and clamping Na_i with nystatin did not prevent the response. These results suggested that the changes in Na pump were not dependent on changes in Na_i but instead that PKC may independently inhibit the Na,K-ATPase, possibly through phosphorylation (34). Inhibition of the apical exchanger, however, is required for a full effect of the hormone on ouabain binding, possibly through a decrease in Na_i concentration, which is rate-limiting for the activity of the Na,K-ATPase (35). It is therefore likely that the effects of PKC on the pump and the CaM kinase II-induced inhibition of Na uptake are additive, with the sum of both actions leading to the 4-fold decrease in ouabain binding (6).

Our results therefore suggest that both the basolateral Na,K-ATPase and the apical NHE are effectors of the CT receptor in a proximal tubule kidney cell line, providing direct evidence that CT can regulate two of the main Na transport systems of the kidney tubule. Although it is known that CT has a potent natriuretic effect (2, 3), attributed for the most part to an inhibition of Na reabsorption in the proximal tubule, no direct evidence for such a mechanism of action of CT has previously been provided. The profound inhibition of the apical NHE and of the Na pump observed here in a kidney cell line could indeed provide a plausible mechanism for the ability of CT to decrease Na reabsorption *in vivo*. On the other hand, the fact that these effects are cAMP independent and therefore observed only during S phase of the cell cycle in this cell line makes it difficult, at that stage, to extrapolate to any physiological responses. It is noteworthy, however, that regions of the kidney tubule have been identified where the cells have a receptor but fail to respond to the hormone with an increase in cAMP (15, 16, 36), showing instead an increase in Ca_i (15).

Finally, it is of interest to compare the effects of CT with those of dopamine and α-adrenergic agonists in the kidney tubule. Like CT, dopamine is a potent natriuretic agent *in vivo*, which inhibits both the Na,K-ATPase (37, 38), via PKC (26, 27), and the NHE (39). However, and in contrast with CT, dopamine concomitantly increases cAMP, which also inhibits the Na,K-ATPase (23). Norepinephrine has effects opposite those of dopamine on both Na,K-ATPase activity and Na excretion (23, 40), and inhibition of the Na,K-ATPase involves an increase in Ca_i and activation of the Ca/calmodulin-dependent phosphatase (calcineurin) (23). It is interesting to note that, in some respects, CT seems to act on the Na,K-ATPase in the kidney cell line LLC-PK_1 (clone 4) as either dopamine or norepinephrine, depending on the position of the cells in their cell cycle, possibly reflecting differential effects of the hormone on various segments of the kidney tubule (16, 36).

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1. Azria, M. (1989) *The Calcitonins* (Karger, Basel), pp. 1–152.
2. Ardaillou, R. (1975) *Nephron* **15**, 250–260.
3. Bijvoet, O. L. M., van der Sluys Veer, J., De Vries, H. R. & Van Koppen, A. T. J. (1971) *N. Engl. J. Med.* **284**, 681–688.
4. Giebisch, G. & Aronson, P. (1986) in *Physiology of Membrane Disorders*, eds. Andreoli, T. E., Hoffman, J. F., Fanestil, D. D. & Schultz, S. G. (Plenum, New York), pp. 669–700.
5. Schultz, S. (1986) in *Physiology of Membrane Disorders*, eds. Andreoli, T. E., Hoffman, J. F., Fanestil, D. D. & Schultz, S. G. (Plenum, New York), pp. 519–534.
6. Chakraborty, M., Chatterjee, D., Kellokumpu, S., Rasmussen, H. & Baron, R. (1991) *Science* **251**, 1078–1082.
7. Pfaller, W., Gstraunthaler, G. & Loidl, P. (1990) *J. Cell. Physiol.* **142**, 247–254.
8. Grinstein, S. & Rothstein, A. (1986) *J. Membr. Biol.* **90**, 1–12.
9. Mahnensmith, R. L. & Aronson, P. S. (1985) *Circ. Res.* **56**, 773–788.
10. Clark, J. D. & Limbird, L. E. (1991) *Am. J. Physiol.* **261**, C945–C953.
11. Yun, C. H. C., Gurubhagavatula, S., Levine, S. A., Montgomery, J. L. M., Brant, S. R., Cohen, M. E., Cragoe, E. J., Jr., Pouyssegur, J., Tse, C.-M. & Donowitz, M. (1993) *J. Biol. Chem.* **268**, 206–211.
12. Malgaroli, A., Meldolesi, J., Zambonin Zallone, A. & Teti, A. (1989) *J. Biol. Chem.* **264**, 14342–14347.
13. Moonga, B. S., Towhidul Alam, A. S. M., Bevis, P. J. R., Avaldi, F., Soncini, R., Huang, C. L.-H. & Zaidi, M. (1992) *J. Endocrinol.* **132**, 241–249.
14. Su, Y., Chakraborty, M., Nathanson, M. & Baron, R. (1992) *Endocrinology* **131**, 1497–1502.
15. Murphy, E., Chamberlain, M. & Mandel, L. (1986) *Am. J. Physiol.* **251**, C491–C495.
16. Chabardes, D., Imbert-Teboul, M., Montegut, M., Clique, A. & Morel, F. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3608–3612.
17. Casavola, V., Helmle-Kolb, C. & Murer, H. (1989) *Biochem. Biophys. Res. Commun.* **165**, 833–837.
18. Haggerty, J. G., Cragoe, E. J., Jr., Slayman, C. W. & Adelberg, E. A. (1985) *Biochem. Biophys. Res. Commun.* **127**, 759–767.
19. Cohen, M. E., Reinlib, L., Watson, A. J. M., Gorelick, F., Rys-Sikora, K., Tse, M., Rood, R. P., Czernik, A. J., Sharp, G. W. G. & Donowitz, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8990–8994.
20. Nichols, R. A., Sihra, T. S., Czernik, A. J., Nairn, A. C. & Greengard, P. (1990) *Nature (London)* **343**, 647–651.
21. Jefferson, A. B., Travis, S. M. & Schulman, H. (1991) *J. Biol. Chem.* **266**, 1484–1490.
22. Yamagata, Y., Czernik, A. J. & Greengard, P. (1991) *J. Biol. Chem.* **266**, 15391–15397.
23. Aperia, A., Ibarra, F., Svensson, L.-B., Klee, C. & Greengard, P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7394–7397.
24. Kleyman, T. R. & Cragoe, E. J., Jr. (1988) *J. Membr. Biol.* **105**, 1–21.
25. Agarwal, N., Haggerty, J. G., Adelberg, E. A. & Slayman, C. W. (1986) *Am. J. Physiol.* **251**, C825–C830.
26. Bertorello, A. & Aperia, A. (1989) *Am. J. Physiol.* **256**, F370–F373.
27. Bertorello, A. M., Aperia, A., Walaas, S. I., Nairn, A. C. & Greengard, P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11359–11362.
28. Lin, H. Y., Harris, T. L., Flannery, M. S., Aruffo, A., Kaji, E. H., Gorn, A., Kolakowski, L. F., Lodish, H. F. & Goldring, S. R. (1991) *Science* **254**, 1022–1024.
29. Crocker, P. R. & Gordon, S. (1986) *J. Exp. Med.* **164**, 1862–1875.
30. Haggerty, J. G., Agarwal, N., Reilly, R. F., Adelberg, E. A. & Slayman, C. W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6797–6801.
31. Chabre, O., Conklin, B. R., Lin, H. Y., Lodish, H. F., Wilson, E., Ives, H. E., Catanzariti, L., Hemmings, B. A. & Bourne, H. R. (1992) *Mol. Endocrinol.* **6**, 551–556.
32. Emmer, E., Rood, R. P., Wesolek, J. H., Cohen, M. E., Braithwaite, R. S., Sharp, G. W. G., Murer, H. & Donowitz, M. (1989) *J. Membr. Biol.* **108**, 207–215.
33. Weinman, E. J., Dubinsky, W. P., Fisher, K., Steplock, D., Dinh, Q., Chang, L. & Shenolikar, S. (1988) *J. Membr. Biol.* **103**, 237–244.
34. Attali, B., Romey, G., Honoré, E., Schmid-Alliana, A., Mattéi, G., Lesage, F., Ricard, P., Barhanin, J. & Lazdunski, M. (1992) *J. Biol. Chem.* **267**, 8650–8657.
35. Weinman, E. J., Dubinsky, W. & Shenolikar, S. (1989) *Kidney Int.* **36**, 519–525.
36. Geppetti, P., Baldi, E., Manzini, S., DelBianco, E., Maggi, C., Natali, A. & Mannelli, M. (1989) *J. Clin. Endocrinol. Metab.* **69**, 491–495.
37. Bello-Reuss, E. Y., Higashi, Y. & Kaneda, Y. (1976) *Am. J. Physiol.* **242**, F634–F640.
38. Aperia, A., Bertorello, B. & Seri, I. (1987) *Am. J. Physiol.* **252**, F32–F45.
39. Felder, C. C., Campbell, T., Albrecht, F. & Jose, P. A. (1990) *Am. J. Physiol.* **259**, F297–F303.
40. Ibarra, F., Aperia, A., Svensson, L.-B., Eklof, A.-C. & Greengard, P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 21–24.