Phorbol ester stimulation of RasGRP1 regulates the sodium-chloride cotransporter by a PKC-independent pathway

Benjamin Ko*, Leena M. Joshi*, Leslie L. Cooke*, Norma Vazque‡, Mark W. Musch*, Steven C. Hebert†‡§, Gerardo Gamba†, and Robert S. Hoover*‡§

*Department of Medicine, University of Chicago, Chicago, IL 60637; †Molecular Physiology Unit, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de Mexico, Tlalpan 14000, Mexico City, Mexico; and ‡Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520

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The sodium-chloride cotransporter (NCC) is the principal salt-absorptive pathway in the mammalian distal convoluted tubule (DCT) and is the site of action of one of the most effective classes of antihypertensive medications, thiazide diuretics. We developed a cell model system to assess NCC function in a mammalian cell line that natively expresses NCC, the mouse DCT (mDCT) cell line. We used this system to study the complex regulation of NCC by the phorbol ester (PE) 12-O-tetradecanoylphorbol-13-acetate (TPA), a diacylglycerol (DAG) analog. It has generally been thought that PEs mediate their effects on transporters through the activation of PKC. However, there are at least five other DAG/PE targets. Here we describe how one of those alternate targets of DAG/PE effects, Ras guanyl-releasing protein 1 (RasGRP1), mediates the PE-induced suppression of function and the surface expression of NCC. Functional assessment of NCC by using thiazide-sensitive Na⁺ uptake revealed that TPA completely suppresses NCC function. Biotinylation experiments demonstrated that this result was primarily because of decreased surface expression of NCC. Although inhibitors of PKC had no effect on this suppression, MAPK inhibitors completely prevented the TPA effect. RasGRP1 activates the MAPK pathway through activation of the small G protein Ras. Gene silencing of RasGRP1 prevented the PE-mediated suppression of NCC activity, the activation of the H-Ras isoform of Ras, and the activation of ERK1/2 MAPK. This finding confirmed the critical role of RasGRP1 in mediating the PE-induced suppression of NCC activity through the stimulation of the MAPK pathway.

Results

mDCT Cells Natively Exhibit NCC Activity. To assess the impact of DAG/PE stimulation on NCC activity, we used mouse DCT [mDCT, a gift of Peter Friedman (University of Pittsburgh, Pittsburgh, PA)] cells for our experiments. These cells demonstrate transport characteristics consistent with cells from the second portion of the mouse and human DCT, including NCC protein expression, amiloride-sensitive sodium uptake, and thiazide-sensitive sodium uptake (8, 9). However, the 22Na⁺ uptake protocols used in those studies involved determining radiotracer uptake while the mDCT cells were in suspension (9). To eliminate any artifact because of exposure to trypsin and to more closely mimic physiological conditions, we developed a protocol to measure the NCC activity of mDCT cells grown in a monolayer. Using this protocol, the mDCT cells demonstrated significant

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3To whom correspondence may be addressed. E-mail: steven.hebert@yale.edu or rhover@medicine.bsd.uchicago.edu.

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uptake was calculated as the difference in radiotracer uptake between the uptake medium and either vehicle or 1 mM metolazone. Thiazide-sensitive were incubated in chloride-free (Cl-free) or chloride-containing (with Cl) compared with vehicle. (from the epithelial sodium channel (ENaC), Na
amiloride, benzamil, and bumetanide to inhibit sodium uptake compared with the chloride-containing group.

Fig. 1. Chloride-dependent and thiazide-sensitive 22Na
uptake of mDCT cells. (A) mDCT cells were preincubated in chloride-free medium and then incubated for 20 min in either chloride-free or chloride-containing uptake medium and increasing concentrations of metolazone (n = 8). *, P < 0.01, compared with vehicle. (B) mDCT cells preincubated in chloride-free medium were incubated in chloride-free (Cl-free) or chloride-containing (with Cl) uptake medium and either vehicle or 1 mM metolazone. Thiazide-sensitive uptake was calculated as the difference in radiotracer uptake between the metolazone-containing and metolazone-free groups (n = 8). *, P < 0.01, compared with the chloride-containing group.

TPA Reduces NCC Surface Expression. The activity of NCC can be regulated by a change in either its surface expression or the intrinsic function of the cotransporter. To explore the role of the former in our cell system, cell-surface biotinylation experiments were performed on mDCT cells incubated with vehicle, TPA, or 4a-PDD. The amount of total NCC protein in the cell lysate was similar in each group (Fig. 2C). TPA significantly reduced the expression of NCC on the mDCT cell surface, whereas 4a-PDD had no significant effect on NCC surface expression. This ~68% reduction in surface expression suggests that the majority of the decrease in activity seen with TPA exposure is mediated by changes in surface expression.

TPA’s Effect on NCC Activity Is Mediated by RasGRP1. To determine the pathway by which TPA exerts its effect on NCC, 22Na
uptakes were performed with TPA in the presence of various inhibitors. None of the inhibitors alone affected NCC function (data not shown). Treatment with bisindolylmaleimide (BIM), an inhibitor of classical and novel isoforms of PKC, did not abolish the effect of TPA on NCC activity in either mDCT cells or X. laevis oocytes [Fig. 3A and supporting information (SI) Fig. 8]. Similarly, Go6976, a classical PKC inhibitor that also blocks the action of PKD, did not change TPA’s effect on NCC activity.

Fig. 2. Inhibition of NCC Activity by TPA. (A) 22Na
uptake of X. laevis oocytes injected with either water (H2O) or cRNA for rat NCC (rat NCC) and treated with 100 μM metolazone (M), 1 μM TPA (T), or DMSO vehicle (C). Data from uptakes performed by using an uptake medium with chloride (NC) are included as a negative control (n = 38). *, P < 0.01, compared with control NC. (B) 22Na
uptake of mDCT cells treated with 150 nM forskolin (FSK), 100 nM TPA, 100 nM 4a-PDD (PDD), or control (DMSO vehicle, Q) before being placed in uptake medium (n = 12). *, P < 0.01, compared with DMSO. (C) Surface proteins of mDCT cells treated as in B were biotinylated. Immunoblotting and densitometry for total lysate NCC protein and surface biotinylated NCC in each group is shown (n = 10). NCC band appears at 110 kDa. *, P < 0.001, compared with control and PDD groups.
Taken together, these data suggest that TPA's effect was not because of the stimulation of an isofrom of PKC or PKD and that TPA acted by binding to another mediator that shared the C1-binding domain. Therefore, the cells were treated with CalC, which blocks the C1 domain. As expected, the addition of CalC prevented any reduction in NCC activity in response to TPA (42.2 ± 5.7 nmol/mg for CalC with TPA vs. not different from zero for TPA alone) (Fig. 3A).

The RasGRP family of proteins is activated by DAg by the C1 domain and activates the small G protein Ras by removing GDP to form active RasGTP. RasGTP ultimately leads to MAPK activation, triggering Raf-1, MEK1/2 (MAP and ERK Kinase 1/2), and finally ERK1/2 MAPK (extracellular signal-regulated kinases 1 and 2) activation. Given this signaling pathway and the fact that RasGRP1 is expressed in the distal tubule of the mammalian kidney (10), we speculated that RasGRP1 was the DAG-binding domain. Therefore, the cells were treated with CalC, which blocks the C1 domain. As expected, the addition of CalC prevented any reduction in NCC activity in response to TPA (42.2 ± 5.7 nmol/mg for CalC with TPA vs. not different from zero for TPA alone) (Fig. 3A).

To confirm the DAG/PE effect, we directly investigated the role of RasGRP1 in this process. As expected, RasGRP1 is clearly expressed in mDCT cells, as seen by immunoprecipitation and subsequent Western blotting and by RT-PCR of mRNA collected from mDCT cells (SI Fig. 9).

To assess the role of RasGRP1 in TPA’s effect on NCC, mDCT cells were transfected with siRNA for RasGRP1 (siRG), nontargeting siRNA (NT), or empty vector (C). Cells were lysed 48 h after transfection, and immunoprecipitation and immunoblotting with antiRasGRP1 antibody were performed (n = 5 for accompanying densitometry). *P < 0.01, compared with C and NT. (B) 22Na uptake experiments were performed on similarly transfected cells treated with 100 nM TPA (T) or vehicle (DMSO) (n = 12). *P < 0.05, compared with C + T or NT + T.

Fig. 4. NCC activity after gene silencing of RasGRP1. (A) mDCT cells were transfected with siRNA for RasGRP1 (siRG), nontargeting siRNA (NT), or empty vector (C). Cells were lysed 48 h after transfection, and immunoprecipitation and immunoblotting with antiRasGRP1 antibody were performed (n = 5 for accompanying densitometry). *P < 0.01, compared with C and NT. (B) 22Na uptake experiments were performed on similarly transfected cells treated with 100 nM TPA (T) or vehicle (DMSO) (n = 12). *P < 0.05, compared with C + T or NT + T.

TGA-Induced Stimulation of RasGRP1 Activates H-Ras. To confirm this finding, we examined the effect of TPA on RasGRP1’s site of action, Ras. Studies were conducted by using an activated Ras pull-down assay on lysates of mDCT cells treated with either nontargeting siRNA or siRNA specific for RasGRP1. TPA was found to increase total Ras-GTP by 16.0 ± 1.5% (P < 0.05) (Fig. 5A). Total Ras-GTP in the RasGRP1-silenced mDCT cells was significantly reduced by 27.3 ± 6.9%, compared with control (P < 0.05). Notably, there are three major isoforms of Ras (H-Ras, K-Ras, and N-Ras), and preferential activation of specific isoforms by various effectors has been described (11). Given the disparity between the magnitude of the increase in RasGTP levels and the dramatic reduction in NCC activity, we therefore examined the Ras activation patterns of each of these isoforms of Ras. The increase in RasGTP was entirely because of increased H-Ras-GTP because activated Ras pull-down assays performed by using isotype-specific antibodies showed a 32.7 ± 4.5% increase (P < 0.01) in H-Ras-GTP with exposure to TPA (Fig. 5B). RasGRP1 silencing with siRNA significantly reduced H-Ras-GTP levels by 90.5 ± 2.1%, compared with control (P < 0.01) (Fig. 5B). No significant change was observed in H-Ras-GTP levels in RasGRP1-silenced mDCT cells treated with TPA. N-Ras and K-Ras showed no alterations in activation with TPA or gene silencing (Fig. 5B). These experiments demonstrate that stimulation of RasGRP1 by TPA in this system specifically and significantly activates H-Ras.

RasGRP1 Mediates Activation of ERK1/2 by TPA. To assess the impact of TPA-mediated RasGRP1 stimulation on ERK1/2 MAPK...
investigating the regulation of NCC by using cells of collecting duct origin transfected with human NCC reported that PEs had no effect on the thiazide-sensitive $^{22}\text{Na}^+$ uptake. We have now established a method for reliably measuring the function of NCC in a physiologically relevant cell system that natively expresses NCC. Investigation of the complex regulation of NCC by DAG/PE using this system demonstrated the suppression of NCC and revealed a new regulatory pathway for the physiological effects of DAG/PE on transport proteins. Given the well described heterogeneity of hormonal response, effectors, and second messengers between different nephron segments (13), the heterologous expression of NCC in cells of collecting duct origin may have played a role in the conflicting results.

Although DAG has been historically linked to the activation of PKC as the sole mediator of its effects, there are at least five other families of proteins (including RasGRP) that are bound and activated by DAG and PEs (7, 14). Using specific inhibitors of PKC and PKD, we were able to demonstrate that these DAG targets are not involved in the suppression of NCC by PE. Because one of the members of the RasGRP family of proteins (RasGRP1) is expressed in the distal tubule of the mammalian kidney (10), we investigated whether it was mediating the PE effect. The RasGRP family of small G proteins also includes RasGRP2, −3, and −4. RasGRP1 and −3 are high-affinity DAG binders (15, 16) that have been implicated in the control of cell differentiation and proliferation, particularly in T and B cells (17, 18). They also appear to play key regulatory roles in keratinocytes and neuroendocrine cells (19, 20). The pathway of DAG/PE activation of RasGRPs is complex and cell-specific. In some cells, the activation of RasGRPs by PE appears to be mediated through PKC (21, 22). In other cells, DAG/PE stimulation of RasGRPs is PKC-independent (19, 20). After activation by DAG, RasGRP1 activates the small G protein Ras by causing release of GDP from Ras. Ras is then free to bind and be activated by GTP (Fig. 7). There are three isoforms of Ras (H-Ras, K-Ras, and N-Ras). Ras activates Raf, the first kinase in the ERK1/2 MAPK pathway. Raf then phosphorylates and activates MEK1/2, which phosphorylates and activates ERK1/2.

Fig. 5. Ras activation in mDCT cells after gene silencing of RasGRP1. mDCT cells transfected with siRNA for RasGRP1 (siRG) or nontargeting siRNA (NT) were transfected in chloride-free medium and then treated for an additional 15 min with 100 nM TPA or vehicle (DMSO). Cells were then lysed, and a RasGTP pull-down was performed, followed by immunoblotting for Ras (21 kDa). Shown are representative immunoblots and densitometries. (A) Ras (all isoforms) (n = 4), * and & $P < 0.01$ as compared to NT. (B) H-, K-, and N-Ras (n = 4), * $P < 0.01$ as compared to NT.

Fig. 6. ERK phosphorylation in mDCT cells after gene silencing of RasGRP1. mDCT cells transfected with siRNA for RasGRP1 (siRG) or nontargeting siRNA (NT) were treated for 15 min with 100 nM TPA or vehicle (DMSO). Cells were then lysed, and immunoblotting for phosphorylated or total ERK1/2 was performed. ERK1 appears at 44 kDa, and ERK2 appears at 42 kDa (n = 4), * $P < 0.01$, compared with NT.

Discussion
The vast majority of work on the regulation of NCC has been carried out in heterologous expression systems. Using the X. laevis oocyte expression system, we found that PEs were potent suppressors of NCC activity (Fig. 2A). However, a study (12)
there is a basal level of ERK1/2 activation that is unaffected by expression clearly prevents this activation (Fig. 6). However, PE exposure is present, and the suppression of RasGRP1 isoforms of Ras are not activated by PE and are not dependent on RasGRP1. The expected significant increase in ERK1/2 activation with activation of ERK1/2 provides additional insight into this pro-

isoform of Ras in mDCT cells, resulting in ERK1/2 activation through RasGRP1, appear to preferentially activate the H-Ras isoform-specific expression and stimulation, we find this preferential localization (25, 26). Given the heterogeneity of Ras isoform-specific expression and stimulation, we tested whether RasGRP1 was preferentially activating specific Ras isoforms. This finding revealed that PEs appear to preferentially activate H-Ras, and that basal and PE-stimulated activation of H-Ras depends on RasGRP1 (Fig. 5). The other isoforms of Ras are not activated by PE and are not dependent on RasGRP1.

An examination of the effect of RasGRP1 silencing on the activation of ERK1/2 provides additional insight into this process. The expected significant increase in ERK1/2 activation with PE exposure is present, and the suppression of RasGRP1 expression clearly prevents this activation (Fig. 6). However, there is a basal level of ERK1/2 activation that is unaffected by the suppression of RasGRP1 expression. This RasGRP1-independent basal level of ERK1/2 activation correlates well with the RasGRP1-independent basal activation of K-Ras and N-Ras. Thus, N-Ras and K-Ras activation through a non-PE, non-RasGRP1 pathway could potentially mediate the basal ERK1/2 activation. There are a number of proteins that are not DAG/PE-stimulated proteins that activate Ras. One of these proteins could be mediating the basal activation of ERK through N-Ras and K-Ras, whereas RasGRP1 mediates the PE-stimulated activation of H-Ras and ERK that regulates surface expression of NCC.

The suppression of NCC function mediated by TPA appears to be primarily because of a decrease in surface expression (Figs. 2 and 3) that depends on MAPK. Acute changes in surface expression are a well-established regulatory mechanism for transport proteins. Presently, the only established mechanism of regulating NCC surface expression is WNK4 inhibition of forward trafficking of NCC (27, 28). This process does not involve dynamin-dependent endocytosis and may involve alterations in lysosomal degradation before plasma membrane insertion. However, there are numerous examples of transport protein regulation through dynamin-dependent endocytosis. MAPKs also have been shown to mediate changes in surface expression of ion transport proteins (29–31). The RasGRP1/MAPK pathway could regulate the endocytotic pathway in NCC, providing a pathway for regulation of NCC surface expression distinct from WNK4 effects on NCC. Further elucidation of the mechanisms mediating these changes in surface expression is necessary.

Although the regulatory impact of DAG/PE on NCC has been demonstrated in two systems here, the question of what triggers the DAG release in the mammalian distal tubule cells remains unanswered. It is well established that certain heterotrimeric G proteins activate phospholipase C, which then releases DAG and inositol (1,4,5)-trisphosphate from the cell membrane (Fig. 7). However, the identity of a hormonal or physiological stimulus that activates a GPCR and regulates NCC has yet to be identified. There is some evidence that aldosterone regulates NCC (32, 33). However, the mineralocorticoid receptor is not a GPCR. Angiotensin II is a GPCR, and there is some evidence that angiotensin II increases the surface expression of NCC (34). However, changes in the function of NCC by angiotensin II have not been demonstrated, and an increase in surface expression is the opposite effect from what we see with activation of RasGRP1. There are numerous otherGPCRs present in the DCT, and there are presently no definitive studies linking these receptors to the function of NCC. Future studies are necessary to define these upstream regulators of this important cotransporter.

We have established a reliable, reproducible system to study the regulation of NCC function in mammalian DCT cells and used this system to correlate function with surface expression. This system allowed us to study the regulation of NCC by DAG/PE, revealing that a regulator of transport proteins, RasGRP1, mediates the effects of DAG/PE in a PKC-independent manner. These findings suggest that we may need to reexamine the effects on transport proteins that have previously been attributed to PKC. Additionally, we have shown that PEs, acting through RasGRP1, appear to preferentially activate the H-Ras isoform of Ras in mDCT cells, resulting in ERK1/2 activation and suppression of NCC function and surface expression (Fig. 7).

Materials and Methods
Assessment of NCC Function in mDCT Cells. mDCT cells grown to 90% confluence in 12-well plates were incubated at 37°C in an isotonic, Cl−-free medium [130 mM Na gluconate, 2 mM/kg gluconate, 1.0 mM Ca gluconate, 1 mM/mg gluconate, 5 mM HEPES/Tris (pH 7.4), 1 mM amiloride, 0.1 mM bumetanide] for 30 min to increase the driving force for radiotracer uptake. During this time period, the cells were treated with the various
agents being studied or vehicle (DMSO). The medium was then changed to a $^{22}\text{Na}^+$-containing medium (140 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES/Tris (pH 7.4), 1 mM amiloride, 0.1 mM bumetanide, 0.1 mM benazapril, 1 mM ouabain, and 1 $\mu$Ci/mL of $^{22}\text{Na}^+$) with or without 0.1 mM metolazone and incubated for 20 min. Tracer uptake was then stopped by washing with ice-cold PBS. Cells were subsequently lysed with 0.1% (wt/vol) SDS. Radioactivity was measured by liquid scintillation, and protein concentrations of the lysates were determined [bicinchoninic acid (BCA) protein assay; Pierce]. Uptakes were normalized to nmol/mg. Thiazide-sensitive uptake was given by the difference of the uptakes with and without thiazide.

**X. laevis Oocyte Preparation and Assessment of NCC Function.** The preparation and assessment of NCC function of *X. laevis* oocytes is described in detail in *SI Materials and Methods.*

**Cell-Surface Biotinylation.** mDCT cells were incubated in Cl$^-$-free medium for 15 min. Then 100 $\mu$L TPA, 4$a$PD, or DMSO (vehicle) was added to the medium for 15 min. The Cl$^-$-free medium was removed, and uptake medium (without $^{22}\text{Na}^+$ agent) was added to the medium for 15 min. Then 100 $\mu$L of biotinylated protein was added for 30 min. The cells were washed with PBS, and cell-surface proteins were labeled, collected, and isolated by using the pinpoint cell-surface protein isolation kit (Pierce). Protein concentrations were determined by using a BCA protein assay kit. For SDS/PAGE analysis, 80 $\mu$L of total protein from cell lysates were loaded on 30 $\mu$L of biotinylated protein from the DMSO control group and the proportionate volume from the rest of the biotinylated protein groups.

**Activated Ras Assay.** mDCT cells grown to 80% confluence after transfection with siRNA were incubated for 30 min in Cl$^-$-free medium. During this time period, the cells were treated with 100 nM TPA for 15 min. Cells were subsequently lysed and affinity-purified for activated Ras by using the EZ-Detect Ras Activation Kit (Pierce). Immunoblotting was then performed by using antibodies for Ras and its major isoforms.

**ERK1/2 Phosphorylation Assay.** mDCT cells grown to 80% confluence after transfection with siRNA were incubated for 30 min in Cl$^-$-free medium. During this time period, the cells were treated with 100 nM TPA for 15 min. Cells were subsequently lysed in 1 mL of lysis. The lysates were homogenized by sonication on ice. Samples were immunoblotted for phosphorylated ERK1/2 and total ERK1/2.

**Other Methods.** The previous methods also used typical uses of cell culture, immunoprecipitation, immunoblotting, transfection of siRNA, and RT-PCR. For complete details regarding these methods, see *SI Materials and Methods.*

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