Previously, we showed that increased extracellular

tonicity promotes increased type A natriuretic peptide

receptor (NPR-A) expression through a p38 MAPKβ

pathway in inner medullary collecting duct cells. The

endothelial and inducible nitric-oxide synthase (eNOS

and iNOS respectively) genes are also expressed in this

nephron segment and are thought to play a role in reg-

ulating urinary sodium concentration. We sought to
determine whether changes in tonicity might regulate

NOS gene expression, and if so, whether these latter
changes might be linked mechanistically to the increase
in NPR-A gene expression. Increased extracellular
tonicity affected a time-dependent reduction in eNOS
and iNOS protein levels, eNOS mRNA levels, and eNOS gene
promoter activity over the first 8 h of the incubation.

Although levels of the eNOS mRNA and promoter activ-
ity had returned to normal after 24 h, eNOS protein
levels remained low at 24–36 h, and recovery was not
complete even at 48 h. The decrease in eNOS expression
was signaled in large part through a p38 MAPK-depend-
ent mechanism. Reduction in eNOS expression together
with the concomitant decline in intracellular cyclic
GMP levels appears to account for a significant portion
of the p38 MAPK-dependent osmotic stimulation of
NPR-A gene expression noted previously. Collectively,
these findings support the existence of a complex regu-
ulatory circuitry in the cells of the inner medullary col-
collecting duct linking two independent cyclic GMP-gener-
signaling transduction systems involved in regulation
of urinary sodium concentration.

Cells of the inner medullary collecting duct (IMCD) play a
unique role in the management of fluid and electrolyte home-
ostasis. Positioned at the most terminal portion of the nephron,

the IMCD deals with up to 5% of filtered sodium load and is
responsible for the final decision regarding urinary sodium
concentration (1). In this pivotal role, the IMCD receives and
integrates information from a number of local and systemic
regulatory factors including mineralocorticoids, prostaglandin
E2, endothelin, interleukin-1, and atrial natriuretic peptide.
IMCD cells by virtue of their location in the inner renal me-
dulla are exposed to tremendous variations in extracellular
tonicity, often extending into the osmolar range (1). Hyperos-
motic stimuli have been shown to regulate gene expression in
IMCD (2–6) as well as non-renal cells (7, 8). Some of these gene
products are linked to cellular adaptation to hyperosmotic stress
(2–4, 7, 8), whereas others appear to be involved in the
regulation of specific physiological processes (5, 6).

IMCD cells also express the three major isoforms of nitric-
oxide synthase (NOS) (9). Increased production of NO in IMCD
cells and subsequent activation of the soluble guanylyl cyclase
have been linked to increased natriuretic activity (10). How-
ever, to date, no information has been published regarding the
effect of prevailing extracellular tonicity on the expression of
NOS mRNA or protein in IMCD.

This study was designed to evaluate the effects of hyperos-

morality on eNOS gene expression in cultured IMCD cells and
determine the relationship of observed changes to the stimula-
tion of NPR-A gene expression noted previously (11). Our find-
ings demonstrate that eNOS expression is reduced, at least
transiently, through a p38 MAPK-dependent mechanism. The
reduction in eNOS expression reduces basal cyclic GMP levels
in these cells, which in turn appears to be linked to the increase
in NPR-A gene transcription, steady-state mRNA levels, and
functional catalytic activity.

MATERIALS AND METHODS

Materials—

An assay kit was purchased from PerkinElmer Life Sci-
fences (La Jolla, CA). 5′-32P-dCTP was purchased from PerkinEl-
erer Life Sciences. eNOS antibody was from Santa Cruz Biotechnology (Santa
Clara, CA). Primer-it® RMT kit, hybridization solution, and NucTrap push columns
were purchased from Qiagen Inc. (Santa Clara, CA). cGMP radioimmunoassay kit
was purchased from PerkinElmer Life Sciences. Primer-it® RMT kit, hybridization
solution, and NucTrap push columns were purchased from Strатеген. Other reagents
were obtained from standard commercial suppliers.

Isolation and Culture of IMCD Cells—Adult Sprague-Dawley rats
were euthanized by CO2 narcosis followed by bilateral thoracotomy in
compliance with a protocol approved by the University of California San
Francisco Committee on Animal Research. Kidneys were excised and
bivalved with a scalpel blade. The inner medullary tissue was dissected
free from the outer medulla, minced into one-cubic millimeter frag-
ments, and digested with 1 mg/ml collagenase at 37 °C with gentle
agitation during each 30-min cycle. IMCD cells were enriched in the

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preparation using hypotonic lysis as described previously (12). The cells were suspended in medium 1 (1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 10% fetal bovine serum, 42 mM sodium bicarbonate, 100 IU/ml penicillin, and 100 μg/ml streptomycin) and seeded on to culture plates. After 24 h, the cells were transfected into medium 1 (3 ml of the modified Eagle’s medium and Ham’s F-12 medium supplemented with 10 mM HEPES (pH 7.4), 42 mM sodium bicarbonate, 5 μg/ml insulin, 50 mM hydrocortisone, 5 μg/ml transferrin, 5 μM triiodothyronine, 100 IU/ml penicillin, and 100 μg/ml streptomycin) and cultured for 3–4 days.

Measurement of Basal and ANP-stimulated cGMP Levels—IMCD cells were grown to ~80% confluence and incubated for different periods of time under conditions outlined in the individual figure legends. For measurement of ANP-stimulated cGMP accumulation, cells were washed three times with prewarmed phosphate-buffered saline and incubated with 0.5 ml of Dulbecco’s modified Eagle’s medium containing 0.5 mM isobutylmethylxanthine and 10 mM HEPES (pH 7.4) for 10 min at 37 °C. 100 μM ANP was added to the medium, and the incubation was continued for another 10 min. The reaction was stopped by the promoter fragment was originally isolated as a BglII/NarI fragment (56). The fragment produced by PCR was cut with BglII terminus. The fragment produced by PCR was cut with

RNA Isolation and Northern Blot Analysis—IMCD cells were plated in 10-cm dishes, cultured, and treated with different reagents as indicated in the figure legends. Total RNA was extracted from cells using the RNAeasy mini kit according to instructions provided by the manufacturer. Total RNA was denatured and separated on a gel containing 2.2% formaldehyde, transferred to a nitrocellulose filter, and hybridized to radiolabeled cDNA probe as described previously (13). A 1.2-kb EcoRI fragment of the rat NPR-A cDNA (13) and a 4.0-kb EcoRI fragment from the bovine eNOS cDNA (14) kindly provided by W. Sessa were isolated from vector sequence, radiolabeled using the primer-itR RMT kit (Stratagene), and separated from free nucleotide using NuCl Trap push column (Stratagene). The membranes were then hybridized with the relevant 32P-labeled cDNA for 1 h at 68 °C and hybridized with the relevant 32P-labeled cDNA for 1 h at 68 °C in hybridization solution provided by Stratagene. All membranes were subsequently stripped and rehybridized with a radiolabeled 1150-bp BamHI/EcoRI fragment of 18 S rDNA to permit normalization among samples for differences in RNA loading and/or transfer to the filter. Hybridization signal was detected by autoradiography and quantified using an image program.

Plasmid Constructions—pcDNA3-p38β and pcDNA3-p38β (AF), a kinase-defective mutant of p38β, which does not display dominant-negative activity in our system (data not shown), were kindly provided by Dr. Jhauai Han of Scripps Research Institute (La Jolla, CA) (15). pcDNA3-MK6KαL, a dominant-negative MK6K mutant, was provided by J. R. Woodgett (University of Toronto, Toronto, Canada) (18). A promoter fragment spanning −1197 to +22 in the human eNOS gene was isolated from genomic sequence provided to us by F. Soubrier (17). Fragment generation was carried out by PCR using an upstream sense oligonucleotide, which incorporated a HindIII site at its 5’ terminus and a downstream antisense oligonucleotide containing a BglII site at its 3’ terminus. The fragment produced by PCR was cut with HindIII and BglII and ligated into the HindIII/BamHI sites of α-luciferase, a luciferase containing plasmid described previously (17). The −1575 to −687 bp of NPR-A promoter fragment was originally isolated as a BglII (5’ terminus)/NarI (3’ terminus) fragment and cloned into the pF0xLuc vector (19). Subsequent studies suggested that pF0xLuc contained cryptic transcriptional regulatory elements that idiosyncratically responded to selected experimental perturbations including exposure to hypertonic medium. To circumvent potential complications in interpretation of experimental data, the pF0xLuc fragment was recloned into the BglII/BamHI sites of α-luciferase, a luciferase containing plasmid described previously (17).

Transfection and Luciferase Assay—Cells were plated in 6-well plates and grown to ~70% confluence. At that time, transfection was carried out with Lipofectin reagent (Invitrogen) using a protocol recommended by the manufacturer. Transfection efficiency (~45%) was estimated by direct visualization of cells 24 h following introduction of RSV-GFP (green fluorescent protein). 1 μg of −1197eNOS-LUC or −1575NPR-A-LUC with 0.2 μg of cytomegalovirus-β-galactosidase was introduced into each well. The DNA-liposome suspension was incubated in the cultures for 5–6 h at 37 °C in Opti-MEMI reduced serum medium (Invitrogen). The suspension was then removed and replaced with K-1 medium for the human cells that were treated with different concentrations of sucrose, NaCl, or urea in K1 medium for defined periods of time. At the end of the incubation, cells were washed three times with phosphate-buffered saline and lysed with Promega lysis buffer. Luciferase activity was measured using the luciferase assay system (Promega). β-Galactosidase activity was assayed using the pNBT-1 chromogenic assay (Tropix, Bedford, MA). Luciferase levels were normalized for β-galactosidase activity in the individual cultures.

Immunoblot Analysis—40 μg of total protein was denatured at 100 °C for 3 min in loading buffer (125 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromphenol blue, 2% β-mercaptoethanol), subjected to 8% SDS-PAGE, and transferred onto polyvinylidene difluoride membrane at 28 V overnight in transfer buffer plus SDS (25 mM Tris (pH 7.5), 190 mM glycine, 20% methanol, 0.05% SDS). The membrane was blocked with 5% nonfat milk in TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) and probed with anti-eNOS (diluted 1:1000 in TBST), anti-iNOS, or anti-nNOS antibody (diluted 1:100 in TBST). A horseradish peroxidase-conjugated secondary antibody (diluted 1:1000 in TBST) was employed to detect immunoreactive bands using ECL Western blotting detection system (Amersham Chemical Corp.). Signal was identified and quantified using NIH Image.

Statistical Analysis—Data were evaluated using one-way ANOVA with Newman-Keuls test for significance.

RESULTS

Hyperosmolality Inhibits eNOS Gene Expression in IMCD Cells—eNOS or NOS-3 is produced at relatively high levels in the inner medullary collecting duct of the kidney (9). In fact, nitric oxide has been suggested to participate in the control of renal function in this and other locations (20, 21) in the kidney. Nitric oxide, like the NPR ligands, also signals a large portion of its functional activity through stimulation of cGMP production in target cells. It does so through the activation of the soluble versus particulate (NPR-dependent) guanylyl cyclase. We have shown previously that cGMP is an effective downregulator of NPR-A gene expression in rat aortic smooth muscle (19) and IMCD (22) cells in culture. We reasoned that changes in eNOS activity and, by inference, NO production might be linked to the osmoregulation of NPR-A.

As shown in Fig. 1A, both sucrose (150 mM) and NaCl (75 mM) but not urea (150 mM) effected a time-dependent reduction in eNOS mRNA levels between 1 and 8 h following application of the osmotic stimulus. Notably, a rebound in expression restored eNOS transcripts to control levels at 24 h despite the continued presence of the osmotic stimulus. Similar findings were observed following introduction of a chimeric eNOS promoter-driven luciferase reporter into IMCD cells prior to the application of osmotic stress. As shown in Fig. 1B, there was a reduction in promoter activity (maximum of 50% at 4 h), which recovered at 24 h following application of the osmotic stimulus. It is noteworthy that the expression of eNOS at the protein level (Fig. 1C), assessed through Western blot analysis, displayed a similar pattern of suppression; however, unlike the transcripts and promoter activity, there was only limited recovery of eNOS protein at 24 h into the study. Only after 36–48 h did eNOS protein levels begin to show evidence of recovery, and even at 48 h, recovery was not complete. The osmotically inactive molecule urea had no effect on either eNOS mRNA or protein levels over the time course of the experiments carried out here.

Both iNOS or NOS-2 and nNOS or NOS-1 are also known to be expressed in the IMCD of the kidney (9). To explore the extent of the osmotic suppression of total NO synthetic capacity, we examined the expression of iNOS and NOS in the IMCD cell culture using exposure to 75 mM NaCl. As
shown in Fig. 2, the levels of iNOS protein were reduced following the application of the osmotic stimulus, paralleling the reduction in eNOS levels noted above. However, the levels of nNOS were unchanged by the increase in extracellular tonicity over the same time interval.

Role of p38 MAPK in Signaling the Osmotic Suppression of NOS Gene Expression—We have shown previously that the osmotic induction of NPR-A gene expression and functional activity is dependent upon an intact p38 MAPK signaling system (11). To examine the potential role of p38 MAPK in promoting the reduction in NOS gene expression in our IMCD cell cultures, we treated cells with SB203580, a selective p38 MAPK inhibitor, for 1 h prior to osmotic stimulation with NaCl. As shown in Fig. 3, a reversal of NaCl-dependent suppression of eNOS protein (Fig. 3A) and promoter activity (Fig. 3B) levels was seen following treatment with SB203580. Similarly, whereas SB203580 had little effect on basal levels of iNOS (Fig. 2), the inhibitor completely reversed the osmotic suppression of this protein. The inhibitor had virtually no effect on nNOS levels in either the presence or absence of NaCl. The forced expression of p38 MAPKβ resulted in the suppression of eNOS gene promoter activity to a level approaching that seen with NaCl (Fig. 3C). The combination of p38 MAPKβ and NaCl were to some degree additive in reducing eNOS promoter activity, whereas a kinase-inactive mutant of p38 MAPKβ (p38β AF) had no effect on the promoter either alone or in the presence of NaCl. Finally, a dominant-negative mutant of MKK6, a kinase responsible for activating p38 MAPK, resulted in the reversal of the NaCl-dependent suppression of the eNOS promoter. Collectively, these findings strongly implicate p38 MAPK as playing a key role in mediating the osmotic suppression of eNOS gene expression.

Role of Cyclic GMP in Mediating the Osmotic Stimulation of NPR-A Gene Expression—As noted above, we have recently demonstrated that increased extracellular tonicity stimulates the expression of the type A natriuretic peptide receptor gene and its functional activity in cultured IMCD cells (11). Given the parallel reduction in NOS with increased extracellular tonicity, we asked whether NO or its downstream second mes-
senger, cyclic GMP, might play a regulatory role in controlling NPR-A expression. More specifically, we reasoned that osmotic inhibition of NOS expression together with the accompanying reduction in cellular cGMP levels might lead to a reflex increase in NPR-A gene expression. We examined basal cyclic GMP levels in cells cultured in the presence of increased cellular osmolality for increasing periods of time. As shown in Fig. 4, both sucrose (150 mM) and NaCl (75 mM) but not urea (150 mM) effected a time-dependent reduction in basal cyclic GMP levels in these cells. The nadir (~60% inhibition) was seen at 24 h following application of the osmotic stimulus. Whether this reduction reflected a loss of endogenous eNOS activity, we would predict that we should be able to mimic the effect of the osmotic stimulus with inhibitors that block signaling through this pathway. As shown in Fig. 5A, L-NAME, a potent inhibitor of eNOS catalytic activity, effected a 40% reduction in basal cGMP levels, a level of inhibition that approached but was not additive with that obtained with NaCl (~50% inhibition). This was accompanied by a doubling of NPR-A mRNA levels (Fig. 5B), a level of stimulation that fell short of that achieved with NaCl alone. The addition of i-NAME to NaCl led to an increase in NPR-A mRNA levels, which was not statistically different from that seen with NaCl alone. This finding suggests that NO does play a role through its cyclic GMP-generating properties in regulating basal NPR-A gene expression, but it cannot account entirely for the NaCl induction of NPR-A expression.

Thus, it appears that a substantial portion of the osmotic stimulation of NPR-A gene expression may be accounted for through the inhibition of the NOS-dependent signaling systems and consequent reduction in cellular cyclic GMP levels. To probe this further, we examined the effects of raising cyclic GMP levels on NaCl-stimulated NPR-A promoter activity in transiently transfected IMCD cells. As shown in Fig. 6, NaCl increased promoter activity ~3–4-fold at 24 h following application of the osmotic stimulus. Under the conditions used in this experiment, neither the NO donor SNAP nor 8-bromo-cyclic GMP significantly altered basal promoter activity. However, when used in combination with NaCl treatment, each of these agents effected ~40–50% inhibition of the osmotic stimulation of NPR-A promoter activity; 8-bromo-cyclic GMP appeared to be slightly more effective in this regard. These findings support our hypothesis that the reduction in cyclic GMP contributes to but once again does not entirely account for the increase in NPR-A gene expression.

Next, we attempted to close the mechanistic loop linking p38 MAPK activation to increased NPR-A gene expression that we
reported previously (11). As shown in Fig. 7A, the NaCl-dependent increase in NPR-A activity (i.e. ANP-sensitive cGMP generation) was almost completely reversed by inclusion of the p38 MAPK inhibitor SB203580 in the culture medium. Transfection of a plasmid encoding the \( \beta/9252 \) isoform of p38 MAPK under conditions that allowed a transfection efficiency of \( \beta/11011 \) 45% (data not shown) resulted in almost a 2-fold increment in NPR-A activity (given the efficiency of transfection, the actual magnitude of the induction is likely to be larger than this.). This effect of P38\( \beta/9252 \) on NPR-A activity was to some degree additive with that produced by NaCl alone and was not observed at all following transfection with the kinase-inactive mutant p38\( \beta/9252 \) AF. Because we have linked the increase in NPR-A activity to a reduction in basal cGMP levels (see Figs. 4–6), we examined the role of p38 MAPK in controlling these levels. As shown in Fig. 7B, whereas SB203580 itself had virtually no effect on basal cGMP levels, it completely reversed the NaCl-dependent reduction in basal cGMP levels. These data support the hypothesis that osmotic stimulation of p38 MAPK activity inhibits eNOS gene expression and its contribution to basal cGMP levels in the IMCD cell. This reduction in cGMP levels is responsible for the subsequent increase in NPR-A expression and activity.

Finally, we turned our attention to the mechanism underlying the changes in eNOS gene expression seen with continued exposure to the osmotic stimulus. As noted in Fig. 1B, the inhibition of the eNOS promoter, which was apparent at 8 h, was lost at 24 h despite the continued presence of NaCl in the culture medium. As shown in Fig. 8, NaCl effected the expected reduction in eNOS promoter activity at 8 h, and this inhibition was not affected by co-incubation with the NO donor SNAP. However, although SNAP alone had no effect on basal eNOS promoter activity after 24 h, it was effective in “restoring” the reduction in promoter activity in the presence of NaCl. Cyclic GMP has been demonstrated to inhibit eNOS gene expression in cultured endothelial cells (23). These data suggest that the “escape” of eNOS gene expression from osmotic suppression at the 24 h time point may result from a secondary reduction in basal cyclic GMP levels (because of a decrease in NO production), effectively neutralizing the cyclic GMP-dependent tonic suppression of eNOS promoter activity.

DISCUSSION

Osmotic regulation of gene expression has been studied extensively in systems ranging from yeast to mammalian cells (24). A significant body of information has been accumulated regarding the signal transduction mechanisms (25) and the transcriptional regulatory controls involved in this process; however, our understanding of osmotic regulation of gene expression remains incomplete. There is little information, for example, to indicate how individual osmoregulated gene products interact with one another to promote alterations in cellular phenotype. This study explores this latter issue for two regulatory proteins expressed in the distal nephron. NPR-A is stimulated (11), while eNOS is suppressed by increased extracellular tonicity. The increase in NPR-A expression appears to result, at least in part, from reduced basal cyclic GMP produc-
tion in cells exposed to increased extracellular toxicity. The reduction of basal cGMP results from inhibition of eNOS gene expression in IMCD cells (Fig. 9).

The osmo-dependent stimulation of NPR-A activity and gene expression raises important questions regarding the role of this stimulation in the regulation of sodium handling in the kidney. Many conditions associated with increased osmolality in the extracellular fluid compartment including that of bathing IMCD cells are associated with contraction of intravascular...
volume, reductions in renal perfusion, and in severe situations, decreased arterial pressure. The reduction in renal perfusion leads to a decrease in medullary blood flow and preservation of medullary hypertonicity. The latter promotes water reabsorption both by increasing the osmotic gradient between collecting duct and medullary interstitium and by suppressing endothelin (5), thereby reducing the vasopressin-antagonizing properties of the latter (26, 27). Urinary sodium is actively conserved along the entire length of the nephron in this setting as the organism struggles to maintain adequate intravascular volume. Under these conditions, it makes sense that those processes leading to sodium excretion, particularly those in the IMCD where the final “decisions” regarding urinary sodium concentration are made, would be suppressed. Hence, reductions in plasma ANP levels (28), which are largely of cardiac origin, and reduced expression of eNOS in IMCD are consistent with the need to promote sodium conservation. Whereas NPR-A levels would be higher in this setting, in the presence of reduced renal and cardiac ANP production, these receptors would for the most part remain unliganded and therefore biologically inert. However, one could conceive of rarer situations where increased extracellular osmolality might be associated with the expansion of the extracellular fluid compartment. In this setting local suppression of endothelin (5) and eNOS (this paper) in the IMCD would work against the perceived need for increased urinary sodium excretion. However, plasma ANP in this setting would predictably be high reflecting the increase in circulating plasma volume. The increased expression of NPR-A in osmo-stimulated IMCD cells would assure continued sodium excretion despite suppression of the local natriuretic systems in IMCD. Thus, stimulation of NPR-A activity/expression in the face of inhibition of other natriuretic mechanisms may provide a “safety valve” function, preserving the ability to maintain urinary sodium excretion in response to systemic natriuretic signals (or increased plasma osmolality) when the endogenous local natriuretic systems are suppressed.

Alternatively, this regulatory system may have evolved to accommodate very specific physiological needs. One example would be the seemingly paradoxical natriuresis that accompanies severe dehydration (29, 30). In this setting, sodium excretion continues in the face of a water deficit (i.e. increased plasma osmolality) and volume contraction. It has been hypothesized that sodium loss is increased in an attempt to restore plasma osmolality toward normal. Increased NPR-A activity as a reflection of increased medullary toxicity could account, at least in part, for this natriuretic activity. We have shown previously that the p38 MAPKβ pathway is the primary pathway signaling the osmotic induction of NPR-A promoter activity (11). A number of studies have demonstrated that p38 MAPK inhibits NOS expression in systems other than IMCD (31–33). The current study links this induction to an upstream stimulus (osmotic stimulation) and downstream effects (reduction in cyclic GMP levels and increased NPR-A expression). The findings reported in this study indicate that at least one component of the p38 MAPK-dependent signal derives from the suppression of basal cyclic GMP levels, implying a mechanistic link between the kinase and those catalytic proteins that control synthesis or degradation of the cyclic nucleotide. The nature of this link remains undefined. Of note, nitric oxide and cyclic GMP have been shown to increase the activity of a MAPK phosphatase 1 in primary cultures of cultured vascular smooth muscle cells (34). A reduction in basal cyclic GMP levels by increased extracellular toxicity might be predicted to reduce activity of MAPK phosphatase 1 and thereby increase the amplitude and/or duration of a MAPK-mediated signal transduction event (i.e. a feed-forward mechanism). In this regard, it will be of interest to determine whether the NaCl-dependent stimulation of p38 MAPK activity is further amplified as cyclic GMP levels begin to fall following exposure to the osmotic stimulus.

Finally, although it is clear that cyclic GMP participates in the osmoregulatory process, it falls well short of accounting for the entire effect. The inhibition of NO-dependent cyclic GMP generation with t-NAME was less effective than NaCl in increasing NPR-A mRNA levels (Fig. 5B). Furthermore, neither 8-bromo-cyclic GMP nor SNAP proved capable of completely reversing the induction of NPR-A promoter activity (Fig. 6). This finding suggests that at least one additional factor independent of cyclic GMP is involved in promoting the p38 MAPKβ-dependent osmotic stimulation of the NPR-A gene (Fig. 9).

Collectively, these data point to a highly complex system for the regulation of gene expression by changes in extracellular tonicity in distal nephron segments. A careful delineation of each component in this system may help to elucidate their respective contributions to the disorders of sodium handling and potentially identify logical targets for therapeutic intervention.

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