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Effect of Efflux of Guanosine 3′,5′ Cyclic Monophosphate (cGMP) on the Regulation of Intracellular Levels of cGMP in the Inner Medullary Collecting Duct

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ABSTRACT. Guanosine 3′,5′ cyclic monophosphate (cGMP) acts as a second messenger in the inner medullary collecting duct (IMCD) where it inhibits sodium transport; therefore, it is important to investigate processes that regulate intracellular cGMP levels. We hypothesized that efflux of cGMP is a major mechanism in this process. IMCDs were isolated from rat kidneys and exposed to atrial natriuretic peptide (ANP) for 0, 3, and 20 min in buffer with or without isobutyl methylxanthine (IBMX), a phosphodiesterase (PDE) inhibitor. Extracellular and intracellular cGMP levels were measured by radioimmunoassay. After cGMP production was stimulated by addition of ANP (10^{-7} M), cGMP efflux was 3.29 ± 0.60 fmol/μg · min at 3 min ($P = 0.016$) and 0.51 ± 0.25 fmol/μg · min at 20 min (NS). Intracellular cGMP peaked at 3 min at 26.66 ± 4.84 fmol/μg ($P = 0.017$) and decreased to 12.98 ± 2.76 fmol/μg at 20 min (NS). Since PDEs were inhibited, these data suggest that efflux regulates intracellular cGMP. Efflux was correlated with intracellular cGMP levels ($r = 0.97$). After 3 min of stimulation with 10^{-9} M ANP, efflux was 2.0 ± 0.3 fmol/μg · min, while intracellular cGMP content was 13.8 ± 3.6 fmol/μg. With 10^{-8} M ANP, efflux was 3.5 ± 0.7 fmol/μg · min, while intracellular content was 20.5 ± 7.6 fmol/μg; and at 10^{-7} M ANP, efflux was 5.1 ± 0.6 fmol/μg · min and intracellular content was 26.6 ± 8.0 fmol/μg. By 20 min, efflux and intracellular levels had returned to control values. Finally, we measured efflux and PDE activity in the absence of IBMX. Efflux was ≈15% of PDE activity (N = 7). We conclude that cGMP efflux is concentration-dependent and, under some circumstances, may be an important regulator of intracellular cGMP levels in isolated IMCDs. BIOCHEM PHARMACOL 53;5:631–636, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cGMP; efflux; sodium transport; ANP; nitric oxide; inner medullary collecting duct

The IMCD†, which forms the terminal segment of the nephron, is responsible for the final regulation of renal handling of sodium and water. The IMCD, therefore, plays a determining role in the regulation of water and sodium excretion and, consequently, overall fluid balance in the body. Changes in sodium absorption in the IMCD occur in response to stimulation of second-messenger signaling systems induced by hormonal agents. The IMCD is one of the major target tissues for the action of ANP [1] and has been shown to respond to NO [2]. Both of these hormones elevate the second messenger cGMP in this segment of the nephron [1, 2]. cGMP inhibits an amiloride-sensitive cation channel in the apical membrane of IMCD cells [3], thereby impairing sodium reabsorption by this nephron segment [4, 5]. Consequently, changes in intracellular IMCD levels of cGMP may contribute to the regulation of sodium in the body.

Intracellular levels of cGMP are a balance between synthesis and removal of cGMP from the cell. Removal of cGMP may be accomplished in two ways: (1) enzymatic breakdown by PDEs and (2) efflux of cGMP out of the cell. Breakdown by PDEs has been studied extensively [6], but there are few studies concerning cGMP efflux. Efflux of cGMP has been shown to occur in a variety of cell types, including MDCK cells [7], vascular smooth muscle cells [8], and liver cells [9], but the ability of the IMCD to export cGMP has not been examined. Furthermore, the relative importance of efflux as compared to degradation in the regulation of intracellular levels of cGMP has not been addressed. The contribution of cGMP efflux to the regulation of intracellular cGMP content also has not been investigated in the IMCD.

We hypothesized that efflux of cGMP is an important mechanism in the regulation of intracellular levels of cGMP in the IMCD. Efflux of cGMP was measured from

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† *Abbreviations:* IMCD, inner medullary collecting duct; cGMP, guanosine 3′,5′ cyclic monophosphate; ANP, atrial natriuretic peptide; IBMX, isobutyl methylxanthine; PDE, phosphodiesterase; NO, nitric oxide; MDCK, dog kidney epithelial cell line; and cAMP, adenosine 3′,5′ cyclic monophosphate.

isolated rat IMCDs stimulated with ANP. Efflux was observed to be dependent upon intracellular cGMP concentration and also constitute approximately 15% of the PDE activity, suggesting that efflux contributes to the regulation of intracellular levels of cGMP and, consequently, sodium transport.

MATERIALS AND METHODS

IMCD Isolation

IMCDs were obtained from Sprague-Dawley rats (110–213 g). On the day of the experiment, rats were anesthetized with ketamine (100 mg/kg body wt, i.p.) and heparinized (10 U/rat, i.p.). The abdominal cavity was opened to expose the kidney, which was perfused *in vivo* with ice-cold buffer solution (mM: 118 NaCl, 4.7 KCl, 0.75 $CaCl_2$, 1.18 NaH_2PO_4, 1.18 $MgSO_4$, 24.8 $NaHCO_3$, 10 glucose) containing 0.2% collagenase I (Sigma, St. Louis, MO) until the effluent was clear of blood. The kidney was removed, and the inner medulla was excised, minced, and subjected to dispersion in the same solution for 60 min. Distilled water (150% by volume) was added, lysing all of the cells except those of the IMCD [10]. The mixture was poured through a 250-μm mesh, and the suspension of IMCDs was washed in buffer to remove the collagenase. IMCD cells and tubules were monitored under phase-contrast microscopy, and viability was assessed by trypan blue exclusion. Numerous tubules were obtained, and viability was roughly 85%.

cGMP Efflux

IMCDs were exposed to various concentrations of ANP for 0, 3, and 20 min in incubation buffer (mM: 114 NaCl, 25 $NaHCO_3$, 2.5 NaH_2PO_4, 4.0 KCl, 1.2 $MgSO_4$, 6.0 alanine, 1.0 trisodium citrate, 5.5 glucose, 2.0 calcium lactate) in the absence or presence of 0.5 mM IBMX at 37°. The extracellular bath was assayed for cGMP accumulation, and the cells were lysed with methanol for determination of intracellular cGMP content. cGMP content was measured by radioimmunoassay (Biomedical Technologies Inc., Stoughton, MA). Recovery was monitored in each experiment and found to be 121 ± 7.1%.

Measurement of PDE Activity

IMCDs were isolated and snap-frozen in homogenizing buffer (mM: 40.0 Tris–HCl, 5.0 $MgCl_2$, 4.0 mercaptoethanol) by submerging the sample tubes in acetone maintained on dry ice. Samples were kept at −70° until the day of the PDE assay. On the day of the assay, samples were thawed and vortexed. PDE activity was measured over 60 min and determined to be linear during this time frame. To determine the dependence of PDE activity on intracellular cGMP concentration, samples were incubated with a range of [^{3}H]cGMP concentrations (0.1 to 2 μM) for 60 min at 37°. Samples were boiled for 3 min to stop the reaction and then loaded onto 0.3 g Al_2O_3 columns. cGMP is eluted from the column while other phosphate guanine compounds are retained [11]. Each column was washed with TES buffer (pH 8.0). PDE activity was determined from the difference between the cGMP added to the sample and that which remained after the 1-hr incubation. These experiments were conducted in the presence of 0.15 μM cAMP because this concentration of cAMP is normally present in the intact cell. cGMP recovery was monitored in each experiment by adding an equivalent amount of [^{3}H]cGMP through a parallel Al_2O_3 micro column and determining the amount that passes into the eluate. Recovery averaged 94 ± 4.0%. In a few experiments, recovery was also monitored by adding [^{32}P]cGMP to the column along with the experimental sample.

Determination of Protein Content and Cell Volume

All measurements were normalized to the sample's protein content. Proteins were determined using Coomassie protein assay reagent (Pierce, Rockford, IL) and measuring absorbance at 595 nm. Bovine serum albumin (1 mg/mL) was used as a standard.

Intracellular volumes of IMCDs were measured using a modified version of the protocol described by Woods and Houslay [7]. Briefly, suspensions of IMCDs in perfusion medium were incubated with [^{3}H]H_2O for 30 min at 37°, such that the intracellular and extracellular [^{3}H]H_2O equilibrated. Then [^{14}C]sucrose was added to the suspension as an extracellular fluid marker. The cell mixture was layered over silicone oil (d = 1.021) on top of 20% trichloroacetic acid (TCA). Samples were centrifuged for 2–3 min. The upper phase and TCA layer were removed for liquid scintillation counting. Intracellular volume was based on [^{3}H]H_2O corrected for extracellular carry-over of fluid as assessed by [^{14}C]sucrose.

Statistics

Experimental results are expressed as means ± SEM. Data were evaluated with a paired *t*-test, with Bonferroni adjustment to determine significance between treatment groups. A ≥95% probability that the means were different was considered significant.

RESULTS

The addition of ANP (10^{-7} M) to IMCDs in the presence of IBMX caused an increase in cGMP efflux over 3 min, which returned to control levels by 20 min (Fig. 1). During the first 3 min, cGMP efflux averaged 3.29 ± 0.60 fmol/μg · min, a 5.5-fold increase compared with the time control in the absence of ANP (0.62 ± 0.12 fmol/μg · min) (P = 0.016; N = 6). Between 3 and 20 min, cGMP efflux in the presence of ANP decreased to 0.51 ± 0.25 fmol/μg · min, not significantly different from efflux in the absence of ANP (0.20 ± 0.06 fmol/μg · min). Accumulation of cGMP in the extracellular bath over time is shown in Fig. 2. Extracellular cGMP accumulation in the presence of ANP

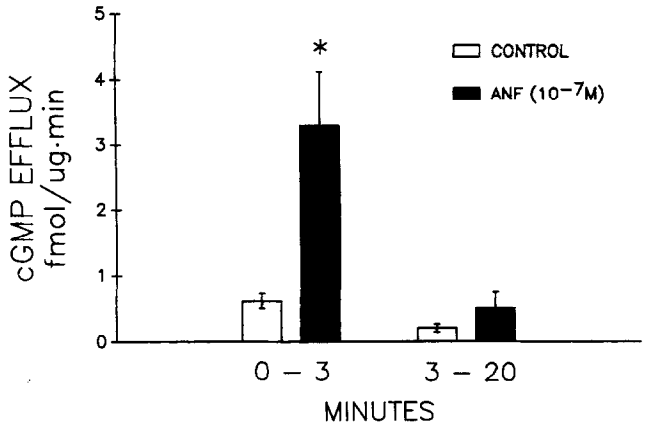

FIG. 1. Efflux of cGMP, in the presence of IBMX, from isolated IMCDs in the presence and absence of ANP over time. Values are means ± SEM, N = 6. ANP (10^{-7} M) caused an increase in cGMP efflux over the initial 3 min (*P = 0.016).

increased from 1.34 ± 0.35 to 11.22 ± 2.03 fmol/μg at 3 min (P = 0.003) and reached 19.94 ± 5.28 fmol/μg at 20 min (P = 0.015). In the absence of ANP, at 3 and 20 min, extracellular levels were 3.20 ± 0.46 and 6.64 ± 1.39 fmol/μg (N = 6), respectively. These data demonstrate that cGMP efflux occurs from intact IMCDs, is elevated within 3 min, and returns to control values by 20 min. To ensure that IBMX was inhibiting PDE activity, IMCDs were incubated with [^{3}H]cGMP in the presence and absence of IBMX. Addition of samples containing only [^{3}H]cGMP to the Al_2O_3 columns resulted in 94 ± 4% recovery of [^{3}H]cGMP in the eluate. IMCDs incubated with [^{3}H]cGMP and IBMX for 60 min gave a recovery of 96 ± 5% [^{3}H]cGMP in the eluate. IMCDs incubated with only [^{3}H]cGMP gave a 47 ± 2% recovery, suggesting that PDE activity accounted for 43% degradation of [^{3}H]cGMP, and that IBMX induced almost complete inhibition of PDE activity.

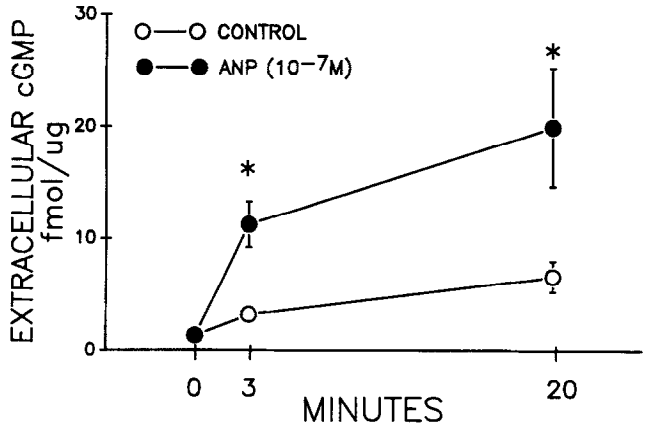

FIG. 2. Accumulation of extracellular cGMP, in the presence of IBMX, from isolated IMCDs in the presence and absence of ANP over time. Values are means ± SEM, N = 6. ANP (10^{-7} M) caused an increase in extracellular cGMP accumulation between 0 and 3 min (*P = 0.003), and between 3 and 20 min (*P = 0.015) compared with basal values in the absence of ANP.

To determine whether efflux was affecting intracellular levels of cGMP significantly, intracellular levels were measured. Intracellular levels of cGMP in the presence of IBMX and ANP (10^{-7} M) also peaked at 3 min at 26.66 ± 4.84 fmol/μg (P = 0.017). After 20 min of exposure to ANP, cGMP levels had decreased to 12.98 ± 2.76 fmol/μg (N = 6; Fig. 3). In the absence of ANP, intracellular cGMP levels decreased slightly (but not significantly) over time from 8.14 ± 1.66 to 4.60 ± 0.95 fmol/μg (N = 6). Accumulated extracellular levels of cGMP (Fig. 2) were comparable to the decrease in intracellular cGMP observed between 3 and 20 min in both the presence and absence of ANP. These data suggest that, in the presence of IBMX and ANP, the increase in efflux at 3 min is returning intracellular levels of cGMP to control values.

Since efflux appeared to change with intracellular cGMP concentration (as noted in the presence and absence of ANP), we examined whether efflux of cGMP correlated with various concentrations of intracellular cGMP by changing the concentration of ANP used to stimulate cGMP production. cGMP efflux increased in a concentration-dependent manner (N = 6; Fig. 4). In the absence of ANP, efflux was 1.50 ± 0.14 fmol/μg · min and intracellular cGMP was 7.14 ± 1.53 fmol/μg. After 3 min of stimulation with 10^{-9} M ANP, efflux was 2.0 ± 0.3 fmol/μg · min, while intracellular cGMP content was 13.8 ± 3.6 fmol/μg. With 10^{-8} M ANP, efflux was 3.5 ± 0.7 fmol/μg · min, while intracellular content was 20.5 ± 7.6 fmol/μg. Finally, efflux was 5.1 ± 0.6 fmol/μg · min with 10^{-7} M ANP and intracellular content was 26.6 ± 8.0 fmol/μg. At 20 min of incubation, intracellular levels of cGMP and effluxes were not significantly different from controls at any concentration of ANP. These data indicate that efflux returns intracellular levels to control values by 20 min; furthermore, the data show that efflux of cGMP is dependent on intracellular levels of cGMP. This is further demonstrated by Fig. 5 (N = 42) which depicts a larger range of intracellular cGMP concentrations and corresponding effluxes measured at 3 min of

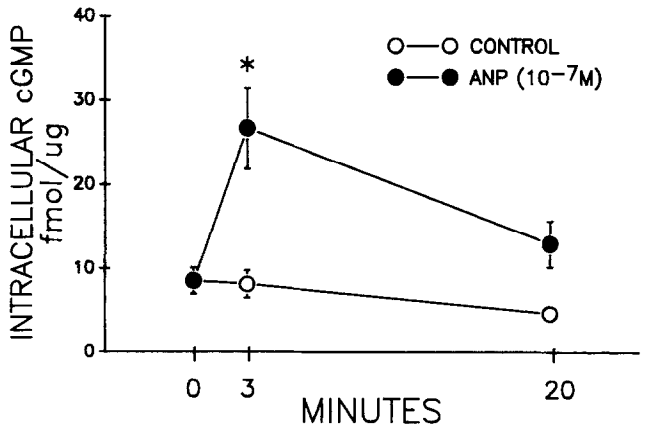

FIG. 3. Intracellular levels of cGMP, in the presence of IBMX, from isolated IMCDs in the presence and absence of ANP over time. Values are means ± SEM, N = 6. ANP (10^{-7} M) caused an increase in intracellular cGMP levels from control at 3 min (*P = 0.017).

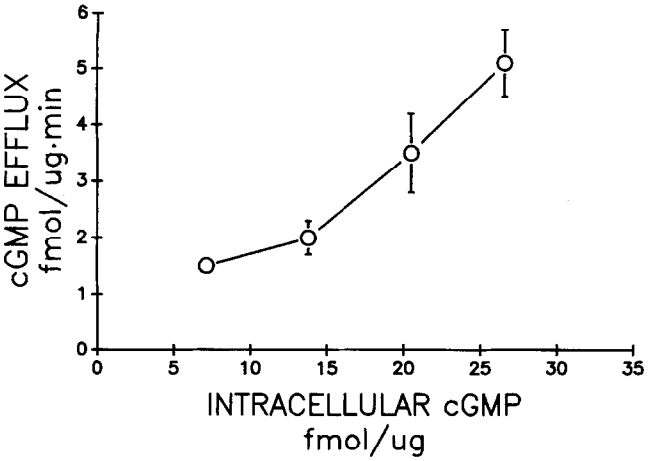

FIG. 4. cGMP efflux in response to increasing intracellular concentrations of cGMP in the presence of IBMX at 3 min. Values are means ± SEM, N = 6.

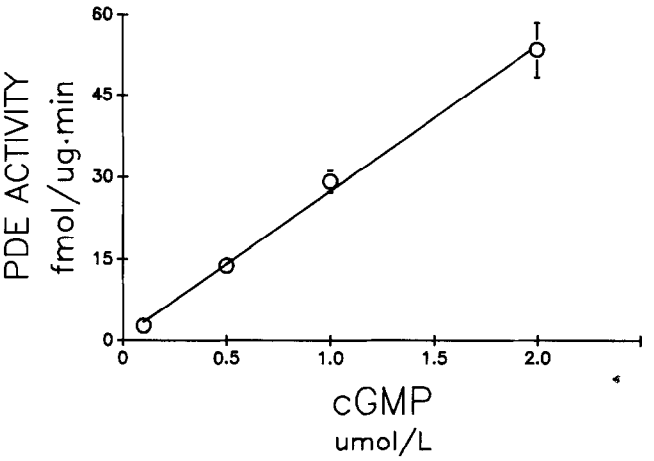

FIG. 6. Concentration–response curve demonstrating the relationship between cGMP and PDE activity. Values are means ± SEM, N = 5 at each concentration. Recovery of PDE was 92%.

incubation with ANP, in both the presence and absence of IBMX. The equation of the line was y = 0.11× + 0.70. These data again depict a concentration-dependent increase in efflux and have a correlation coefficient of 0.84.

Since the rate of efflux appeared to be dependent on intracellular cGMP levels, we compared the contribution of efflux with that of PDE activity in regulating intracellular levels. First, we examined the dependence of PDE activity on intracellular cGMP concentration. Increasing concentrations of cGMP resulted in concentration-dependent increases in PDE activity (Fig. 6). The equation of the line was y = 26.7x + 0.76, with a correlation coefficient of 0.99. Since these experiments were conducted in the presence of 0.15 μM cAMP (described in Materials and Methods), PDE activity was also measured in the presence and absence of cAMP. PDE activity was 3.17 ± 0.33 fmol/μg · min in the absence of cAMP, and 2.98 ± 0.44 and 3.05 ± 0.38 fmol/μg · min in the presence of 0.15 and 1.5 μM cAMP, respectively. cAMP did not appear to inhibit PDE activity under these circumstances.

To determine the relative contribution of efflux to the removal of cGMP from the cell in relation to PDE activity, efflux and intracellular cGMP were first measured at 3 min of incubation with ANP (10^{-7} M) in the absence of IBMX. The measured intracellular cGMP content was then used in the subsequent PDE assay. Efflux was 0.32 ± 0.14 fmol/μg · min at 3 min in the absence of IBMX (N = 7). Intracellular levels of cGMP increased from 0.33 ± 0.06 to 1.95 ± 0.2 fmol/μg at 3 min. Measurement of IMCD protein content and intracellular volume was 67.1 ± 10.8 μg/μL. The intracellular concentration of cGMP, therefore, was 0.13 ± 0.01 μM. PDE activity measured at a cGMP concentration equivalent to that of the measured intracellular concentration of 0.13 μM was 2.2 ± 0.42 fmol/μg · min (N = 6). Consequently, efflux was 15% of the PDE activity.

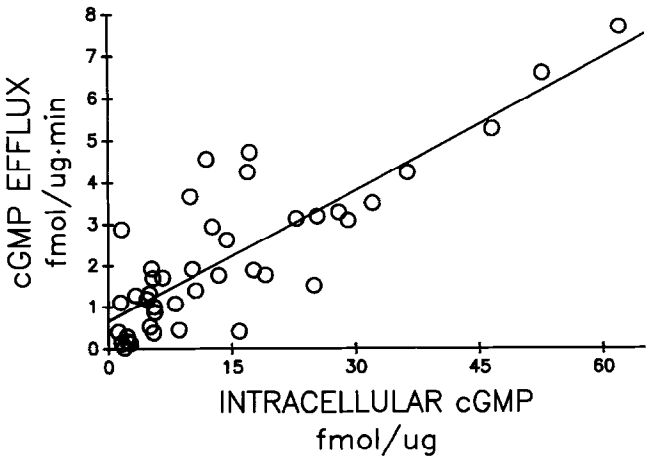

FIG. 5. cGMP efflux in response to various concentrations of cGMP, measured at 3 min of incubation with ANP, in both the presence and absence of IBMX. The line of linear regression has a correlation coefficient of 0.84 (N = 42).

DISCUSSION

This is the first study demonstrating that, in the presence of PDE inhibition, the mechanism of efflux removes cGMP from ANP-stimulated IMCDs until intracellular cGMP levels return to basal values. Furthermore, this study is the first to compare the relative importance of efflux to enzymatic breakdown of cGMP in the regulation of intracellular cGMP levels. Efflux accounts for approximately 15% of the total removal of cGMP from ANP-stimulated IMCD cells. These data suggest that efflux of cGMP may be an important regulator of intracellular levels of cGMP in the IMCD.

Changes in cGMP levels in the IMCD are important because cGMP can inhibit the amiloride-sensitive sodium channel in the apical membrane [3]. This inhibition by cGMP has been shown to occur by a dual mechanism: a direct phosphorylation-independent effect of cGMP and a cGMP-dependent protein kinase [12]. Consequently, cGMP-induced inhibition of the sodium channel can induce

changes in natriuresis and hence the sodium balance in the body.

In the present studies, cGMP efflux demonstrated the capacity of efflux to regulate intracellular levels of cGMP in the IMCD. Efflux decreased ANP-elevated intracellular cGMP levels from 27 to 13 fmol/μg. The decrease in intracellular cGMP levels may be due to efflux, PDE activity, down-regulation of ANP receptors, or desensitization of ANP stimulation of guanylyl cyclase. It is most likely that this decrease in intracellular cGMP is attributed to cGMP efflux because these measurements were made in the presence of IBMX. Essentially, IBMX completely inhibited PDE activity (the recovery of [^{3}H]cGMP in the presence of IBMX and IMCDs was 96 ± 5%). Furthermore, accumulated extracellular levels of cGMP (Fig. 2) were comparable to the decrease in intracellular cGMP observed between 3 and 20 min. Thus, although down-regulation of receptors or desensitization of ANP-stimulated guanylyl cyclase is possible, it seems likely that the lowered cGMP levels in the cell are the result of efflux. The efflux-induced removal of cGMP was important because efflux lowered intracellular cGMP levels back to basal values. The importance of the contribution of efflux to regulation of intracellular cGMP is further shown by the linear increase in cGMP efflux with increasing intracellular concentrations of cGMP. These data are supportive of those shown in other cell types demonstrating the importance of efflux as a regulatory mechanism to eliminate intracellular cGMP. In atriopeptin-stimulated MDCK cells, for example, blockade of efflux with probenecid resulted in a 2.5-fold increase in intracellular levels of cGMP [7]. In vascular smooth muscle cells, PDE inhibition doubled ANP-stimulated accumulation of cGMP in the extracellular bath, indicating an increase in efflux [8]. Similarly, the release of cGMP from stimulated hepatocytes increased 3-fold in the presence of PDE inhibition [9]. These data support the hypothesis of this study that efflux is an important regulatory mechanism for controlling cGMP levels within the IMCD.

This study demonstrates that efflux and PDE activity both contribute to the maintenance of intracellular levels of cGMP, and suggest that under normal circumstances PDE activity is the predominant mechanism for removal of cGMP from the cell. Efflux appears to be responsible for ≈15% of the total removal of cGMP from the cell; the important question becomes how important is this 15%. A 15% elevation in intracellular cGMP has the potential to significantly inhibit sodium absorption in the IMCD. Furthermore, the estimate that efflux is 15% of PDE activity is the lower extreme. Efflux of cGMP is likely to be higher, given that cGMP transporters are localized to the membrane where guanylate cyclase produces cGMP via ANP. PDEs, on the other hand, are compartmentalized to various cytoplasmic regions of the cell. When PDE activity is measured, the compartmentalization and concentration gradients that exist in the cell are destroyed because the assay requires lysing of the cell. Therefore, measured PDE activity for a given intracellular cGMP concentration is probably overestimated. Even if efflux accounts for only 15% of the removal of cGMP from the cell, this still suggests a significant potential for efflux to contribute to regulation of intracellular levels. This contribution becomes much more significant when PDE activity is inhibited. Administration of methylxanthines, such as caffeine, theophylline, or theobromine, to the body would result in PDE inhibition. These compounds are commonly taken for treatment of asthma and dyspnea, and consumed in coffee, tea, and chocolate. Methylxanthines are distributed into all body compartments and have an extremely long half-life ranging between 20 and 50 hr [13] Methylxanthines in combination with stimulated synthesis of cGMP (such as by ANP or NO) may represent a situation where efflux becomes the predominant mechanism for intracellular regulation of cGMP levels.

Understanding the mechanism of cGMP efflux in the IMCD is particularly important because the IMCD is one of the major target tissues for the action of ANP [1, 14]. ANP binds to a 130 kDa receptor in the IMCD which is coupled to guanylate cyclase [15], and results in an increase in cellular cGMP content. The NO donor, sodium nitroprusside, has also been shown to elevate cGMP in IMCD cells [2]. Hormones such as ANP and NO, therefore, regulate blood pressure by inducing natriuresis in the IMCD of the kidney via cGMP, as well as by altering vascular resistance. Consequently, understanding the regulation of cellular cGMP levels may be critical to comprehending blood pressure regulation. In support of this hypothesis, it has been shown that systolic blood pressure is correlated inversely with cGMP levels in the plasma [16], and cGMP in the plasma has been proposed as a clinically useful marker for ANP action [17]. It has also been shown that differing levels of cGMP in IMCD cells could represent a factor contributing to the development of salt-sensitive hypertension in rats [18]. Appel and Dunn [18] demonstrated that IMCDs from prehypertensive Dahl salt-sensitive rats were hyporesponsive to ANP and sodium nitroprusside in relation to intracellular cGMP levels compared with IMCDs from Dahl salt-resistant rats. Thus, these investigators concluded that an aberration in the regulation of intracellular cGMP levels may contribute to the development of salt-sensitive hypertension. An opposite effect, an elevation in cGMP levels in response to ANP, has been shown in glomeruli of spontaneously hypertensive rats compared with normotensive strains [19]. Thus, an aberration in the regulation of cGMP levels associated with hypertension may be tissue- and species-specific.

The efflux of cGMP from IMCD cells is a mechanism that contributes to regulation of intracellular cGMP levels. Efflux of cGMP will result in the removal of all stimulated production of cGMP from the cell during PDE inhibition, demonstrating the capacity of its function. Even in the presence of PDE activity, efflux still contributes to the removal of intracellular cGMP. The process of efflux in the

IMCD is important because it may contribute to the regulation of sodium excretion and consequently blood pressure.

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