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Regulation of atrial natriuretic peptide-stimulated cGMP production in the inner medulla

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Regulation of atrial natriuretic peptide-stimulated cGMP production in the inner medulla. Studies were performed to examine the regulation of atrial natriuretic peptide- (ANP) stimulated guanylate cyclase in the the inner medulla. Primary cultures of rat inner medullary collecting tubular cells exposed to 10^{-7} M ANP increased cGMP formation to 31.2 ± 1.8 compared to the basal production of 2.1 ± 0.6 fm/μg protein. This response did not appear to be transduced via a G_i protein, as preincubation with pertussis toxin did not alter the response to 10^{-7} M ANP, and saponized cells exposed to 10 μM GTPγS did not enhance the response to ANP (77.3 ± 5.9 vs. 86.7 ± 6.3 g/μg). Likewise, changes in extracellular Ca^{2+} from 0.5 to 3.0 mM, decrements in intracellular Ca^{2+} with EGTA or increments in intracellular Ca^{2+} with ionomycin (5 μM) did not significantly alter the response to ANP. Neither activation of protein kinase A with forskolin (36.5 ± 5.1) nor of protein kinase C with s,n-1,2-dioctanoylglycerol (33.2 ± 2.5) altered the response to 10^{-7} M ANP (32.2 ± 3.3, NS). As the inner medullary environment was hypertonic, the effect of altering tonicity was studied. Cells grown for 48 hours in hypertonic media (600 mOsm/kg H_2O) displayed enhanced response to 10^{-8} and 10^{-7} M ANP when osmolality was raised by either Na^+ alone or in combination with urea, but not by urea alone. Our studies demonstrate that ANP-stimulated guanylate cyclase is insensitive to alterations in either intra- or extracellular Ca^{2+}, is not subject to inhibition by protein kinase, and does not involve a pertussis-sensitive G protein. The response is highly sensitive to elevations in tonicity, with Na^+ as the mediator of this effect.

It is now well established that atrial natriuretic peptide (ANP) stimulates guanylate cyclase to produce cGMP in microdissected collecting tubules [1], as well as in fresh suspensions [2] and cultured [3] inner medullary collecting tubule cells. Although this may not be the only signalling system stimulated by ANP in these cells [4, 5], there is considerable evidence to support the view that cGMP mediates the effect of ANP to inhibit Na^+ reabsorption in this segment of the nephron [6, 7]. Therefore, the factors that regulate the response to ANP and its interaction with other signalling systems prevalent in the inner medulla can impact on the cellular effect of the hormone. While the kinetics of the guanylate cyclase and its cation dependence have been well defined [2], other factors that could control the accumulation of cGMP in this tissue remain undefined. The experiments reported herein were therefore performed to assess the role of G proteins, Ca^{2+}, protein kinases, and osmolality on ANP-stimulated cGMP formation in cultured rat inner medullary collecting tubule (RIMCT) cells.

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Methods

Cell culture

Primary culture of RIMCT cells were prepared from male Sprague-Dawley rats as previously described from this laboratory [8]. The morphologic [8], histochemical [9], and more recently scanning electron microscopy [10] suggested a uniform population of principal cells. Despite the fact that the entire papilla was employed in the initial preparation, the cultured cells morphologic appearance of all cells was that of the principal cell. Papillary tissue from three rats was used per 24 well tray. Experiments were performed at 72 to 96 hours in a non-confluent state when each well contained 40 to 75 μg of protein.

Incubation with effector solutions for determination of cGMP

Studies were performed in Krebs-Ringer buffer at the desired Ca^{2+} concentration, containing 0.5 mM isobutyl methyxanthine (IBMX) to inhibit phosphodiesterase, and the effector of interest as previously described [8]. An incubation of 10 minutes was allowed for cGMP generation. Effectors requiring longer exposure prior to ANP stimulation of cGMP were exposed to cells during a preincubation period also with Krebs Ringer but in the absence of IBMX. Except for experiments designed to study the effects of osmolality, the buffer was isotonic. As studies employing GTPγS require cell permeabilization, they were performed in a buffer designed to simulate the intracellular milieu (composition in mmol: 20 NaCl, 100 KCl, 5 mg sulfate, 1 NaH_2PO_4, 25 $NaHCO_3$, 1.0 EGTA, pH 7.2), containing Saponin (50 μg/ml). Such treatment does not interfere with the cell's hormonal responsiveness. Hyperosmolality studies were performed by exposing the cells to a hypertonic environment (600 mOsm/kg H_2O) for 48 hours before the study. This was accomplished by either the addition of 150 mM NaCl, 300 mM urea or a combination of equimolar Na^+ and urea. At the conclusion of the 10 minute incubation with ANP, 0.3 ml of 0.01 N HCl was added to lyse cells and liberate cGMP. cGMP was measured by radioimmunoassay (Amersham International, Arlington Heights, Illinois, USA) in a manner identical to that reported for cAMP [8]. Cellular protein is determined by the method of Lowry et al [11]. Results are expressed as fm cGMP/μg protein. At all times values were obtained in duplicate wells and averaged.

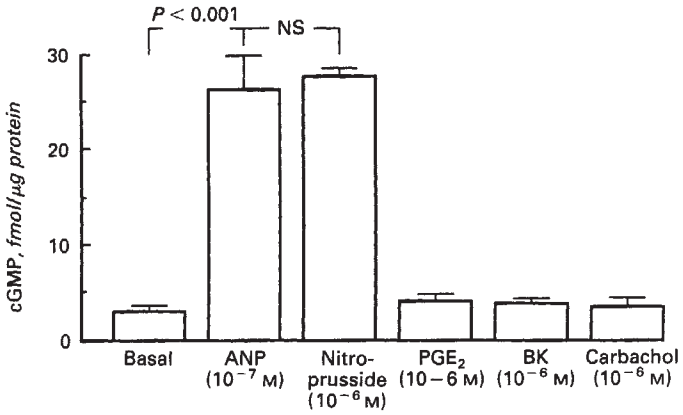

Fig. 1. *Hormonal survey of cGMP response in cultured RIMCT cells.* Only ANP and nitroprusside are associated with increase cGMP formation ($N = 3$).

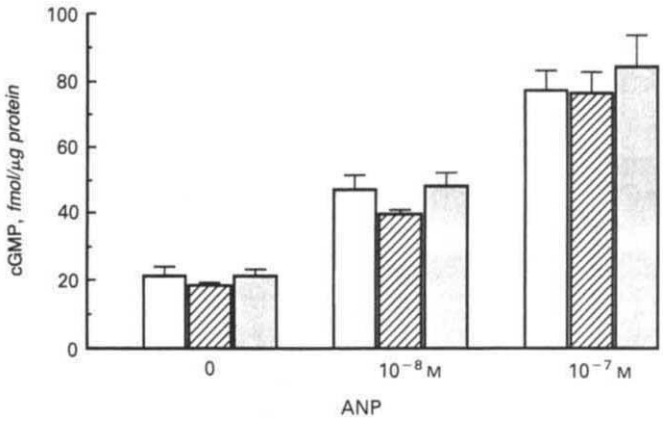

Fig. 2. *Effect of GTPγS and pertussis toxin on ANP-stimulated cGMP production.* Symbols are: (□) control; (▨) pertussis toxin ng/ml/16 hr; (▩) GTPγS 10 μM. Note that GTPγS and pertussis toxin treated cells have a response comparable to that of controls both in the presence and absence of ANP ($N = 4$). No changes reached statistical significance.

Statistical analysis

Comparisons between the treatment groups were done by the unpaired Student's *t*-test. Comparison between three or more groups employed the one-way ANOVA followed by appropriate controls of a prior assumption [12]. A P value of 0.05 was considered significant. Data were presented as mean ± SEM.

Results

cGMP generation by cultured RIMCT cells

In six experiments, the dose-response of cultured RIMCT cells to ANP was studied. No increment was observed at doses $<10^{-9}$ M. At 10^{-8} M cGMP increased from 2.1 ± 0.6 to 10.6 ± 1.7 fm/μg protein and at 10^{-7} M to 31.2 ± 1.8 fm/μg protein. No further increment was noted at 10^{-6} M ANP. To ensure that the response to ANP was specific, the ability of other substances to stimulate cGMP in these cells was investigated. As is shown in Figure 1, only ANP and nitroprusside resulted in generation of the cyclic nucleotide while a number of other agents including prostaglandin E_2, bradykinin and carbachol failed to do so.

Studies on the role of guanine nucleotide binding proteins

To assess whether, as is the case with adenylate cyclase, ANP-stimulated guanylate cyclase activity is transduced by an α_s G protein, the effect of the non-hydrolyzable GTP analogue, GTPγS (10 μM), that enhances the function of G proteins, was studied in saponin permeabilized cells in four experiments. Figure 2 depicts the fact that GTPγS altered neither basal nor ANP-stimulated cGMP formation. Thus, in these permeabilized cGMP, levels rose from 21.2 ± 3.3 to 47.7 ± 2.0 at 10^{-8} M ANP and 77.3 ± 5.9 fm/μg protein at 10^{-7} M ANP, which were levels not different from those seen in GTPγS treated cells (21.4 ± 3.1, 48.2 ± 2.9, and 86.7 ± 6.3 fm/μg protein, respectively). Since some hormonal responses in RIMCT, such as those mediated via phospholipase A_2 [13] and phospholipase C [14], are activated by pertussis-sensitive α_i G protein, we examined the ability of ANP to stimulate cGMP formation in cells pretreated overnight with pertussis toxin (10 ng/ml) for 16 hours, which fully ribosylates susceptible G protein in these cells. As also depicted in Figure 2 in four experiments, the pretreatment with pertussis toxin altered neither basal nor ANP-stimulated cGMP production at either 10^{-7} or 10^{-8} M ANP concentrations.

Studies on the role of extra- and intracellular Ca^{2+}

To determine whether acute changes in Ca^{2+} concentration of the media bathing to RIMCT cells alter the response to ANP, we compared physiologic Ca^{2+} (1 mM) with a low (0.5 mM) and a high (3 mM) Ca^{2+} concentration in three experiments following a 60 minute preincubation with these concentrations of Ca^{2+}. As the top of Figure 3 depicts, neither the increase nor a decrease significantly altered the response. We have previously shown that these alterations in extracellular Ca^{2+} do not change cellular Ca^{2+} concentrations [8]. To effect alterations in cell Ca^{2+}, we exposed the cells to 0.3 mM Ca^{2+} and 1.0 mM EGTA, resulting in a free [Ca^{2+}] of 0.7 μM in order to decrease and to 5 μM ionomycin in order to increase cell Ca^{2+}. As depicted in the bottom of Figure 3, when compared to the response seen at 1 mM Ca^{2+} neither of the maneuvers significantly altered cGMP generation. Neither the slight decrement seen in the presence of EGTA nor the slight increase noted with ionomycin achieved statistical significance.

Studies on the role of protein kinases

Since activation of protein kinases have been found to alter cellular second messenger responses, including phospholipase A_2 [13] and adenylate cyclase [15] as well as phospholipase C [5, 14], we investigated whether guanylate cyclase is subject to similar feedback control. cGMP generation was therefore measured in the presence and absence of the diacylglycerol analogue, s,n-1,2-dioctanoylglycerol. As is depicted in Figure 4, the formation of cGMP in control is unchanged when compared to cell pretreated s,n-1,2-dioctanoylglycerol both in the absence and presence of two concentrations of ANP (10^{-8} and 10^{-7} M). To assess whether stimulation of protein kinase A regulates guanylate cyclase, we studied ANP-stimulated cGMP generation in the presence of 10^{-7} M forskolin. As was the case with protein kinase C stimulation, the generation of cGMP was

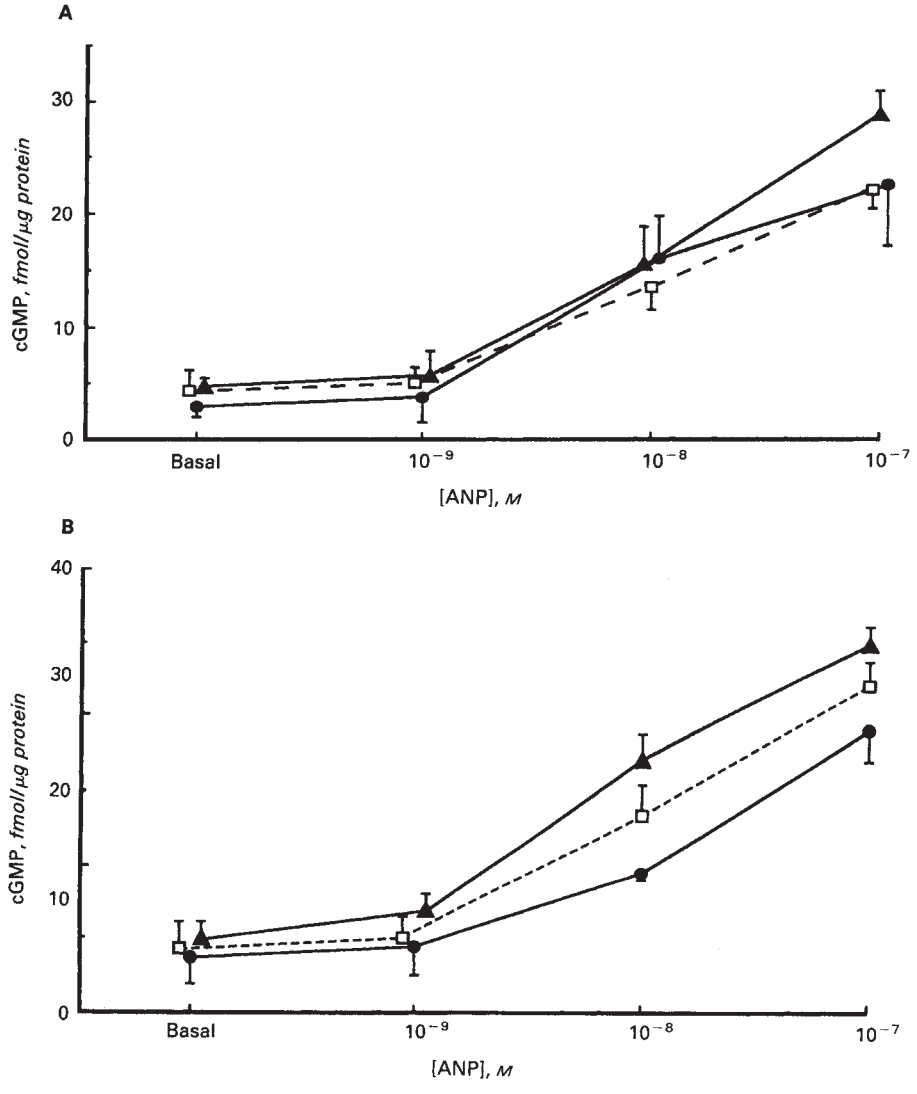

Fig. 3. *Effect of changes in extracellular Ca^{2+}* (**A**) (–●–, *0.5 mM*; --□-, *1.0 mM*; –▲–, *3.0 mM*) *and intracellular Ca^{2+}* (**B**) (–●–, *EGTA*; –■–, *Ca^{2+} 1.0 mM; inomycin 5 μM*) *on the dose-response to ANP* ($N = 4$). No changes reached statistical significance.

unaltered by forskolin both in the presence and absence of 10^{-8} and 10^{-7} M ANP (Fig. 4).

Effect of hypertonicity of cGMP formation

The effect of increasing tonicity on ANP-stimulated cGMP formation was studied, as such increases have been described to affect other hormonal responses including adenylate cyclase [16] and phospholipase C [17]. Also, to prevent acute effects of increased tonicity on cell volume and to allow for the generation of organic solutes a 48 hour exposure was allowed. As shown in Figure 5, hypertonicity enhanced the response to ANP without altering basal cGMP levels. Thus, while in these six experiments 10^{-8} and 10^{-7} M ANP increased cGMP levels to 17.4 ± 3.4 and 34.3 ± 3.6 fm/μg protein, respectively, in cells grown in isotonic media, in cells exposed to 600 mOsm/kg H_2O (300 mM Na^+) the levels rose to 49.5 ± 3.0 and 69.2 ± 4.5 fm/μg protein, respectively, both $P < 0.001$. No such increase was noted when the osmolality was increased to the same level with urea (25.9 ±

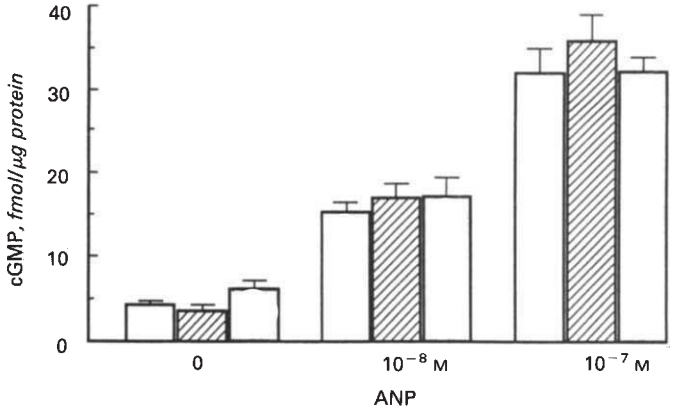

Fig. 4. *Effect of stimulation of protein kinase C with 10^{-7} M s,n-1,2-dioctanoylglycerol (shaded bars) and protein kinase A with 10^{-7} forskolin (striped bars) on ANP-stimulated cGMP production.* Neither altered cGMP production when compared to control cells (open bars) ($N = 4$).

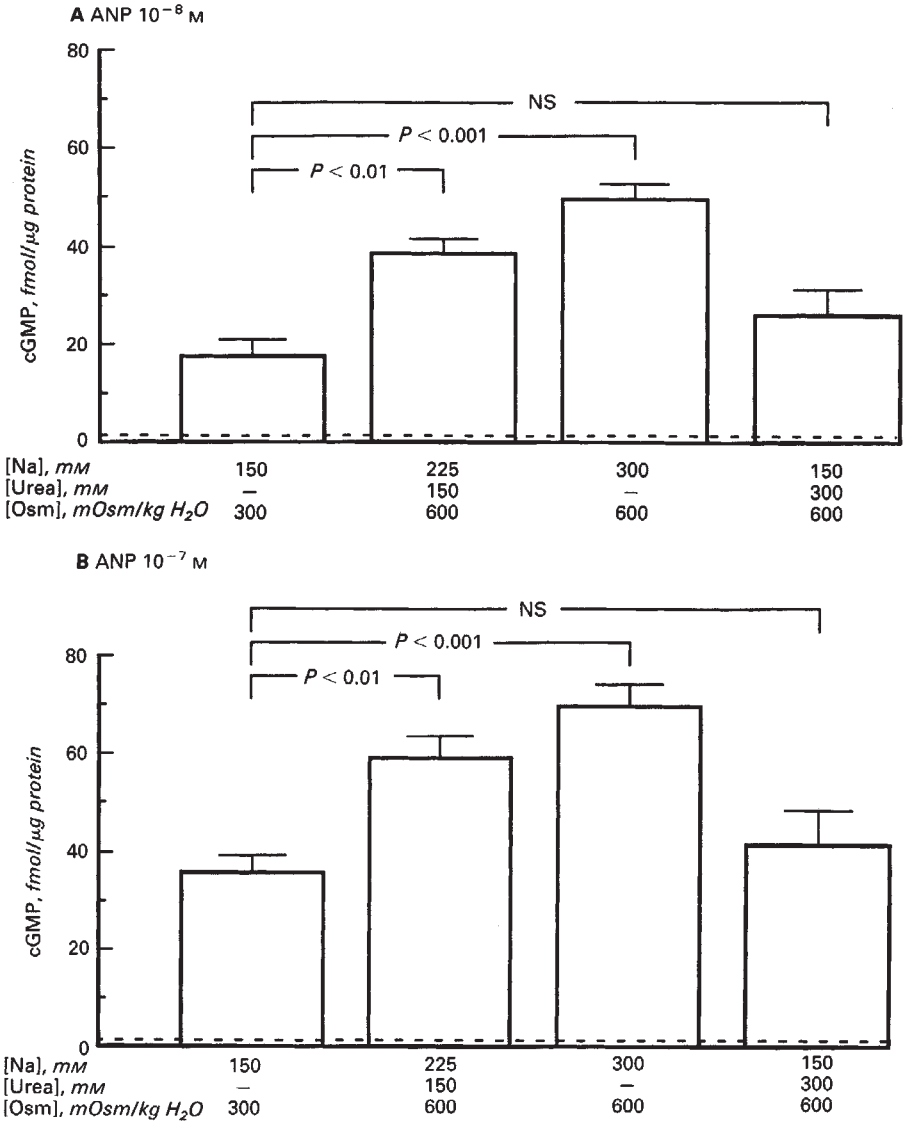

Fig. 5. *Effect of exposure to hypertonicity 48 hours on ANP-stimulated cGMP production.* At both 10^{-8} (**A**) and 10^{-7} M ANP (**B**) the response was enhanced in the presence of either Na^+ alone (third bar) or Na^+ with urea (second bar) but not by urea alone (fourth bar) when compared to isotonic conditions (first bar). The basal cGMP was unaltered and is depicted by the dotted lines ($N = 6$).

5.2 at 10^{-8} M ANP and 41.0 ± 7.1 fm/μg at 10^{-7} M ANP), neither significantly different to control isotonic conditions. Finally, to mimic a situation akin to that prevailing in vivo, when osmolality was raised by both Na^+ and urea, cGMP levels rose to 38.4 ± 3.0 fm/μg at 10^{-8} M ANP ($P < 0.01$ vs. control) and to 59.1 ± 4.1 fm/μg at 10^{-7} M ANP ($P < 0.01$ vs. control).

Discussion

ANP shares with the family of sperm stimulatory peptides a unique ability to stimulate guanylate cyclase and generate cGMP [18]. Although the peptide appears to have receptors at various segments of the mammalian nephron [19], its major site of action appears to be the terminal collecting duct [20]. Since 8-bromo cGMP mimics the effect of ANP to inhibit Na^+ uptake [6], the processes that control and alter the production of cGMP in the inner medulla could greatly impact the final control of Na^+ excretion. The present experiments were designed to explore some of the factors that could impact the accumulation of cGMP as well as to investigate whether activation of other hormone signalling pathways alters the response to ANP in these cells.

Our initial experiments demonstrate that the formation of cGMP is a specific response to ANP, not shared by a number of other hormones that activate other signalling pathways. However, in addition to possessing a particulate form of guanylate cyclase, the comparable response to nitroprusside reflects the presence of the soluble form of the enzyme as well. Signalling mechanisms involving both activation and inhibition of adenylate cyclase [9], activation of phospholipase A_2 [13], and phospholipase C [14] all appear to be transduced by G proteins. In contrast, the activation of guanylate cyclase appears to be entirely independent, at least of the G_i subgroup, of these proteins as neither the exposure to GTPγS nor pretreatment of the cells with pertussis toxin altered the response to ANP. We were unable to test the inhibitory effect of GDPβS because this G protein analogue, unlike GTPγS, cross reacted with the

cGMP immunoassay. Our observations are in agreement with the preliminary results reported by Gunning, Golper and Brenner who also found that ANP-sensitive guanylate cyclase is not regulated by a pertussis-sensitive G protein in MDCK cells [21]. The fact that the enzyme itself appears to act as the peptide receptor may well obviate the need for an intermediate transducing mechanism [22]. It must be noted, however, that other actions of ANP involved G proteins, including its stimulation of phospholipase C in RIMCT cells [5]. ANP inhibition of cAMP formation involving clearance receptors [23] or an effect of cGMP-stimulated phosphodiesterase in others [24] also has been found to be mediated by G proteins. It appears, however, that the cGMP generating role of ANP is independent of G_i protein activation.

We have found variable effects of Ca^{2+} on enzymes involved in signal transduction in RIMCT cells as adenylate cyclase is inhibited and phospholipase C is enhanced by increments in cellular Ca^{2+}. Both enzymes are insensitive to changes in extracellular Ca^{2+} [8, 14]. Such an insensitivity to either a decrease or increase in extracellular Ca^{2+} is evident in guanylate cyclase activation as well. However, in contrast to other systems, we found that maneuvers that decrease or increase cell Ca^{2+} (EGTA and ionomycin, respectively) produced insignificant changes in ANP-stimulated cGMP production. In analyzing the cation dependence of particulate guanylate cyclase, Gunning et al [2] also found a stimulatory effect of Mg^{2+} and particularly Mn^{2+}. In agreement with our results, an increase in extracellular Ca^{2+} concentration from 1 to 4 mM did not inhibit guanylate cyclase; however, in contrast with our results, EGTA did. The possibility that the concentration of EGTA they employed (4 mM) may have chelated other divalent cations to an extent greater than that employed in our study (1 mM EGTA in 0.3 mM Ca^{2+}) must be considered. In the interpretation of the studies employing ionomycin, it must be noted that this ionophore increases cell pH in RIMCT cells [25]. Under different alkalinizing conditions, this effect of increased pH has been reported to enhance the response to 10^{-11} M ANP [26]. An effect of high cell Ca^{2+} to inhibit cGMP generation could thus be obscured. This possibility seems unlikely since in the latter study the enhanced effect of alkalinization was not observed in the background of phosphodiesterase inhibition, conditions akin to those of the present study. It would appear, therefore, that the collecting tubule guanylate cyclase should not be viewed as either a Ca^{2+} stimulated or inhibited enzyme.

Activation of protein kinase C has been shown to decrease both phospholipase C [5, 14] and adenylate cyclase [15] responsiveness in cultured RIMCT cells. Likewise, activation of protein kinase A inhibits phospholipase C activity [15] and probably phospholipase A_2 in these cells [27]. Whether similar mutually inhibitory pathways between signalling mechanisms is also operant vis-a-vis guanylate cyclase has not been previously explored. Our results strongly suggest that this enzyme is not subject to inhibition by either of these kinases, as their activation in a manner that we have previously shown to inhibit other enzymes failed to inhibit ANP-stimulated cAMP formation. In this regard, our results are in disagreement with the observation of Ballerman, Marala and Sharma [28], who noted that protein kinase C inhibited guanylate cyclase activity in membranes obtained from glomerular preparations. Our failure to observe an inhibition is not likely to be due to the lack of preincubation, as this highly permeable analogue as shown to inhibit vasopressin-stimulated cAMP formation by a mechanism reversible with a protein kinase inhibitor without any preincubation [15]. It is possible that the inhibition by protein kinase C is not operant in epithelial cells or that results obtained from enzyme assays performed in a membrane preparation cannot be extrapolated to in situ cGMP generation.

RIMCT cells are uniquely exposed to a large range of osmolalities. The possibility that changes in tonicity could alter hormonal responses has been well recognized. For example, cAMP generation in response to arginine vasopressin is increased [16] and conversely IP_3 formation in response to carbachol decreased [17] as osmolality rises. Gunning et al [2] have examined the effect or toncity on guanylate cyclase activity with variable results. Thus, high Na^+ concentration but not high urea concentration appeared to inhibit guanylate cyclase, but only when the membranes were incubated at 0.5 mM Mn^{2+} and not at 5 mM Mn^{2+}. The authors recognized the limitation of studying this phenomenon in broken membrane preparations, and we therefore examined it in intact cultured cells. To further enhance the relevance of our observations we studied the response to ANP after a 48 hour exposure to the hypertonic environment, a time at which organic osmolytes have accumulated [29] and alterations in cell volume are likely to be minimal. Our experiments reveal that as is the case with cAMP generation, cGMP formation is also enhanced in this setting. This effect appears to be mediated by Na^+ but not by urea. Furthermore, the enhancement is observed when mimicking the in vivo setting by allowing both solutes to contribute to the hypertonic state. The significance of this observation in the control of medullar Na^+ reabsorption in water depleted states will require further investigation.

In summary, in the present study we have investigated some of the factors that could regulate the production of cGMP by ANP in the inner medulla. It is clear that in marked contrast to other signalling pathways in these cells that this process is not transduced by G proteins, is insensitive to both alterations in intra- and extracellular Ca^{2+}, and is not subject to feedback inhibition by stimulation of either protein kinase C nor cyclic nucleotide dependent kinase. Only a prolonged increment in environmental tonicity with Na^+ enhances the hormone's action. With this exception the enzyme appears, therefore, to be regulated by few, if any, other cellular or environmental factors.

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