Korean J. of Pharmacology
Vol. 25, No. 1, pp. 59-66, 1989

Effects of Loop Diuretics on Guanylate Cyclase in Rat Medullary Thick Ascending limb of Henle's Loop

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ABSTRACT

To determine the relation between cGMP and ion reabsorption in rat medullary thick ascending limb of Henle's loop (mTALH) the effects of loop diuretics, furosemide and ethacrynic acid, on the guanylate cyclase of rat mTALH were investigated. The interactions between loop diuretics and cyclooxygenase inhibitors, aspirin and indomethacin, on guanylate cyclase of rat mTALH were also investigated. Furosemide and ethacrynic acid increased guanylate cyclase activity and these effects were not inhibited by aspirin or indomethacin. Arachidonic acid potentiated the stimulatory effect of furosemide on guanylate cyclase. These results suggest that furosemide and ethacrynic acid activate guanylate cyclase directly, and in addition, furosemide affects indirectly via prostaglandin. The reabsorption of sodium chloride may be, at least partially, controlled by cGMP in mTALH.

Key Words: Medullary thick ascending limb of Henle's loop, cGMP, Furosemide, Ethacrynic acid, Prostaglandins

INTRODUCTION

The thick ascending limb of Henle's loop (TALH) is relatively water impermeant, and the salt reabsorption in this segment reclaims 15~20% of the NaCl filtered at the glomerulus. Many investigators have been actively evaluating the specific transport mechanisms involved in NaCl reabsorption by the TALH and the origin of the transepithelial voltage in that segment. There is a general consensus among investigators (Greger, 1981; Hebert *et al.*, 1981; Schlatter & Greger, 1985) that the active reabsorption of NaCl is driven by the action of the Na^+-K^+-ATPase located in the basolateral membrane and sodium is cotransported across the apical membrane together with Cl^- and K^+ by the electroneutral (1 Na^+, 1 K^+, 2 Cl^-) cotransport system. Potassium recycles completely across the potassium conductive apical membrane, and chloride leaves the cell via a conductive pathway in the basolateral membrane. This model for active NaCl reabsorption in the TALH has been proposed originally for the cortical TALH (cTALH) (Greger, 1981; Greger & Schlatter, 1983a; Greger & Schlatter, 1983b) and was strongly supported by results obtained in the isolated medullary TALH (mTALH) and in membrane vesicles of mTALH cells (Eveloff & Kinne, 1983; Hebert & Andreoli, 1984b; Hebert *et al.*, 1981; Hebert *et al.*, 1984; Koening *et al.*, 1983). However, the cTALH and mTALH are heterogeneous with respect to the factors that control NaCl reabsorption (Hebert & Andreoli, 1984a). In the mTALH of some mammalian species, these factors appear to provide a number of feedback loops that sense interstitial osmolality and provide a means of controlling the magnitude of interstitial hypertonicity, and antidiuretic hormone (ADH), PGE_2 and adenosine-3′,5′-monophosphate (cAMP) have been proposed as them.

Hall and Varney (1980) established that in mouse mTALH, ADH increases the spontaneous lumen-positive transepithelial voltage and, concomitantly, the net rate of chloride absorption without affecting the water permeability of that segment. Schlatter and Greger (1985) proposed that the stimulation of active NaCl reabsorption in the mTALH of the mouse by ADH, mediated via cAMP, increases primarily the basolateral chloride conductance. In contrast, while ADH increases cAMP production in mTALH of rat, mouse and rabbit, no effect of pharmacological concentrations of ADH on either the transepithelial voltage or tracer chloride efflux could be demonstrated in rabbit mTALH by Sasaki and Imai (1980). PGE_2, the major product of prostaglandin synthesis in the renal medulla, attenuated

the ADH-dependent NaCl transport in mouse mTALH and this effect was restored by addition of cAMP (Culpepper & Andreoli, 1983).

Lee and Cho (1983), and Lee *et al.*, (1985) proposed that the change in renal tissue level of cGMP related to the action of furosemide and thereby cGMP might act as a mediator or modulator of transport mechanisms involved in NaCl absorption by the ascending limb of Henle's loop. However, these were not sufficient to interpret the role of cGMP in NaCl absorption of mTALH. In the present study, the effects of loop diuretics, furosemide and ethacrynic acid, on the guanylate cyclase of mTALH were investigated. Also, in order to study the relation of prostaglandin to these effects, the interactions between loop diuretics and cyclooxygenase inhibitors, aspirin and indomethacin, on guanylate cyclase of mTALH were investigated.

MATERIALS AND METHODS

Isolation of renal tubules

Male Sprague-Dawley rats (250~300 g) were used in this experiments. The animals were anesthetized with secobarbital 30 mg/kg i.p. and kidneys were perfused with ice-cold saline via the renal arteries. The kidneys were quickly removed and longitudinally cut into thin slices with a razor blade. The slices were placed in ice-cold solution A (Table 1) previously gassed with 95% O_2-5% CO_2 for 30 min. All the following process were performed at 0~4℃ and are described in Fig. 1. The inner stripe of the outer medulla was carefully excised from the brownish outer stripe. The pieces of inner stripe tissue were then forced through a stainless sieve (opening 1×1 mm) with a spatula in order to obtain small cubes of tissue of fairly uniform size. These cubes were submitted to enzymatic digestion in 15 ml of solution A containing 320 U/ml collagenase and 600 U/ml hyaluronidase and vigorously shaken for 1 hr at 37℃ while being continuously bubbled with 95% O_2-5 % CO_2. After the digestion period, the enzyme solution was filtered through a stainless sieve (opening 0.1×0.1 mm) and then the fitrate was centrifuged at 144 g for 2 min and the supernatant

Table 1. Composition of solutions used in this study

	A	B	C	D
NaCl	115	1150	115	115
$NaHCO_3$	25	250	25	25
NaH_2PO_4	2	20	2	2
KCl	5	50	5	5
$CaCl_2$	1			1
$MgSO_4$	1	0.5	0.05	1
Lactate	4			
Alanine	1			
Mannitol	25	350	35	30
Glucose	5			10
Dextran	0.6%			0.6%

All concentrations are in mM unless otherwise indicated.

Osmolarity of all solutions, except B, ranged between 325 and 330 mOms.

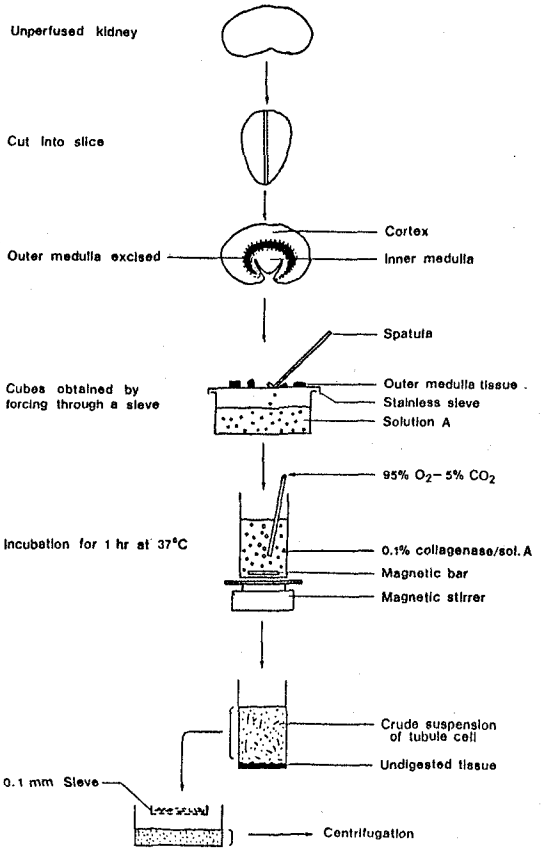

Fig. 1. The different steps of the medullary thick ascending limb cell isolation procedure.

was discarded. The pellet was suspended in 50 ml of ice-cold solution A, placed in an ice bath, and stirred magnetically for 10 min. The suspension was centrifuged at 144 g for 2 min. The pellet was resuspended in solution A and centrifuged again. The pellet was then resuspended in a small volume (0.4~0.6 ml) of solution A.

Purification of renal tubules

Percoll was made isotonic by diluting one part solution B (Table 1) with nine parts Percoll. The isotonic Percoll was then diluted with solution C (Table 1) to yield a 55% Percoll suspension. The Percoll suspension was bubbled with 95% O_2-5% CO_2 and 11 ml were combined with 0.4~0.6 ml tubule suspension and placed in a 12 ml centrifuge tube. The tubes were centrifuged at 20,000 g for 30 min at 4℃. The gradient density was measured using gradient-density marker beads. The tissue which was separated on the resulting continuous density gradient into three distinct regions was respectively collected with a pipette. The preliminary transmission electron microscopy indicated that the material suspended in densities of 1.062 ~1.075 g/ml was enriched in thick ascending limbs. Therefore, this fraction was diluted with solution D (Table 1) and centrifuged at 144 g for 2 min. The pellets were resuspended in solution D and centrifuged and rinsed twice and stored on ice until use.

Assay of guanylate cyclase

The tubules were homogenized in ice-cold homogenizing buffer (0.25 M sucrose, 20 mM tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5). The homogenate was centrifuged at 105, 000 g for 60 min. The supernatant (2-3 mg protein/ml) was used for assay of guanylate cyclase activity. Protein was determined by the method of Lowry *et al.,* (1951) using bovine serum albumin as standards.

Guanylate cyclase activity was estimated from the conversion of [^{3}H]GTP into [^{3}H]cGMP. The assay medium contained 50 mM Tris-HCl (pH 7.6),10 mM theophylline, 15 mM creatine phosphate, 20 μg creatine phosphokinase (100~150 units/mg), 5 mM $MgCl_2$, 0.2 mM GTP, 1 μCi [^{3}H] GTP (1.6×10^{-6} dpm) and enzyme preparation (40 ~50 μg protein) in a final volume of 100 μl. The reaction was run at 37℃ for 10 min and stopped by addition of 100 μl of stopping solution (2% dodecylsulfate, 40 mM GTP and 1.4 mM cGMP) and heating at 90℃ for 2 min. The tracer of [^{14}C] cGMP (20,000 cpm) was added to reaction mixture for recovery determination. GTP was separated from cGMP according to the modification of a method of Craven and DeRubertis (1976), consisting of a double filtration on Dowex and alumina columns. The 2 ml effluent of the alumina column was collected in scintillation vials and 12 ml of scintillation cocktail (toluene 667 ml, triton X-100 333 ml, PPO 5.5 g, POPOP 0.1 g) added. The radioactivity of the vials was counted on ^{3}H and ^{14}C channels in a Packard TRI-CARB 300C scintillation counter. The guanylate cyclase activity was expressed as pmoles cGMP/mg protein/10 min.

Statistics

All values were expressed as the mean±S.E.. The difference between means was assessed for significance by Student's t test. A p value less than 0.05 was considered to represent a significant difference.

Chemicals

Chemicals were of the highest grade available. Compounds purchased from Sigma were: ethacrynic acid, indomethacin, arachidonic acid (sodium salt). PGE_2, $PGF_{2\alpha}$, Dowex AG 50 W×4, alumina (WN-3, neutral), guanosine-5′-triphosphate, guanosin-3′, 5′-cyclic monophosphate, collagenase (type IV), hyaluronidase (type II), creatine phosphate, creatine phosphokinase, theophylline, and dithiotreitol. Furosemide was obtained from Han-Dok Remidia Co. (Korea) and aspirin from Young-Jin (Korea). [8-^{14}C] guanosine-3′, 5′-cyclic monophosphate and [8-^{3}H] guanosine-5′-triphosphate (ammonium salt) were purchased from Amersham (England). Percoll and density-marker bead were purchased from Pharmacia (Sweden).

RESULTS

Effects of furosemide and ethacrynic acid on guanylate cyclase in mTALH were examined at concentrations of 10^{-5} M~10^{-3} M. Furosemide stimulated significantly guanylate cyclase at con-

Table 2. Effect of furosemide and ethacrynic acid on guanylate cyclase in rat mTALH

	Enzyme activity (pmoles cGMP/mg protein/10 min)			
	Furosemide	n	Ethacrynic acid	n
0	8.761±0.456	15	8.635±0.502	7
1×10^{-5} M	10.290±0.647	12	10.511±0.724*	12
1×10^{-4} M	12.017±0.814**	12	13.722±0.810***	12
1×10^{-3} M	14.297±0.931***	12	15.544±0.804***	12

Each value represents the mean±S.E.. n: number of experiments
*$p<0.05$ **$p<0.01$ ***$p<0.001$

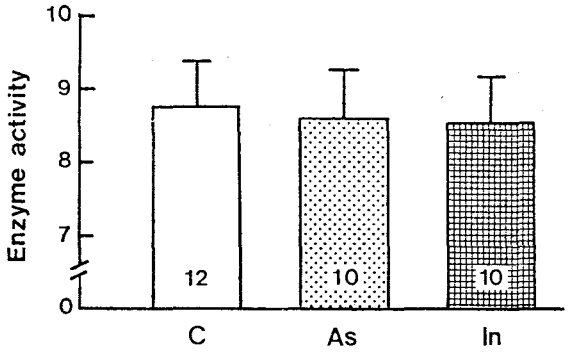

Fig. 2. Effects of aspirin and indomethacin on guanylate cyclase in rat mTALH.
Each value represents the mean±S.E..
Enzyme activity is expressed as pmoles cGMP/mg protein/10 min.
Figure in each bar is number of experiments.
C: Control
As: Aspirin 5×10^{-4} M
In: Indomethacin 5×10^{-4} M

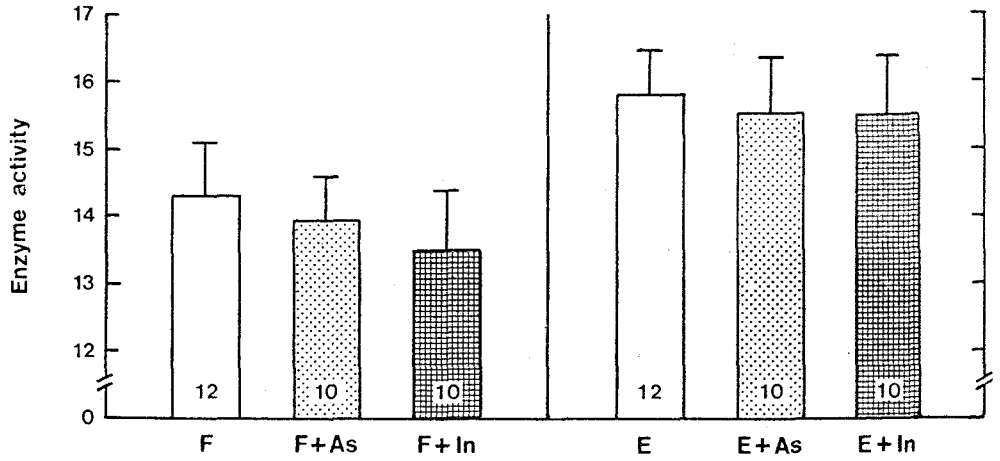

Fig. 3. Effect of aspirin and indomethacin on the effect of furosemide and ethacrynic acid on guanylate cyclase in mTALH.
Each value represents the mean±S.E..
Enzyme activity is expressed as pmoles cGMP/mg protein/10 min.
Figure in each bar is number of experiments.
F: Furosemide 1×10^{-3} M
E: Ethacrynic acid 1×10^{-3} M
As: Aspirin 5×10^{-4} M
In: Indomethacin 5×10^{-4} M

centrations of 10^{-4} M and 10^{-3} M, and ethacrynic acid at concentration of 10^{-5} M, 10^{-4} M and 10^{-3} M (Tabel 2). Aspirin or indomethacin did not affect on guanylate cyclase (Fig. 2) and inhibit the effects of furosemide and ethacrynic acid on guanylate cyclase (Fig. 3). Arachidonic acid (1×10^{-4} M) potentiated the effect of furosemide on guanylate cyclase and this effect was blocked by aspirin (5×10^{-4} M) and indomethacin (5×10^{-4} M). The stimulatory effect of ethacrynic acid was not affected significantly by arachidonic acid (Table 4). PGE_2 and $PGF_{2\alpha}$ increased the guanylate cyclase activity of mTALH (Table 5).

DISCUSSSION

Because of the great diversity of cell types in the mammalian kidney, in vivo experiments are difficult to interpret the interaction between ion transport and metabolism in mTALH, so several types of in vitro preparations have been employed to study the metabolism of the mTALH.

Experiments using slices have been performed on the outer medulla (Lee & Peter, 1969). However, these experiments must be viewed with caution because slices of kidney tissue can have anoxic regions that could lead to misunderstanding of aerobic metabolism (Balaban *et al.*, 1980). So, in vitro studies on well defined isolated nephron segments or cells have been necessitated. Microdissection of individual segments is an elegant technique but is time consuming and requires the subsequent use of sophisticated biochemical microtechniques because of the extremely small amount of tissue obtained. Alternative techniques providing large amounts of pure, well defined nephron segments or cell types are highly desirable. Recently several intestigators (Eveloff *et al.*, 1980; Chamberlin *et al.*, 1984; Trinh-Trang-Tan *et al.*, 1986) developed the new techniques using enzymatic digestion by collagenase for the isolation of mTALH. In this study, we used mTALH cells obtained through the enzymatic digestion by collagenase and hyaluronidase and the gradient centrifugation.

Table 3. Effect of arachidonic acid on guanylate cyclase in rat mTALH

Drug	Emzyme acitivty	n
Control	8.761±0.456	15
1×10^{-6} M	8.795±0.508	10
1×10^{-5} M	8.922±0.574	10
1×10^{-4} M	9.186±0.675	9

Each value represents the mean±S.E..
n: number of experiments
Guanylate cyclase activity is expressed as pmoles cGMP/mg protein/10 min.

Table 5. Effects of prostaglandins on guanylate cyclase in rat mTALH

Drug	Enzyme activity	n
Control	8.761±0.456	15
PGE_2	20.253±0.732***	8
$PGF_{2\alpha}$	21.538±0.823***	8

Each value represents the mean±S.E..
n: number of experiments
Enzyme activity is expressed as pmoles cGMP/mg protein/10 min.
***$p<0.001$

Table 4. Effect of furosemide, ethacrynic acid and arachidonic acid on guanylate cyclase in rat mTALH

	Enzyme activity (pmoles cGMP/mg protein/10 min)			
	Furosemide	n	Ethacrynic acid	n
alone	14.297±0.931	12	15.544±0.804	12
+Ara	17.048±0.924*	12	16.627±0.876	12
+Ara+As	14.813±0.784	10	-	
+Ara+In	14.688±0.811	10	-	

Each value represents the mean±S.E.. n; number of experiments
*$p<0.05$
Ara : Arachidonic acid 1×10^{-4} M
As: Aspirin 5×10^{-4} M
In: Indomethacin 5×10^{-4} M

Furosemide and ethacrynic acid inhibit the reabsorption of NaCl in the TALH, and this effect is due to the inhibition of Cl^- transport which resulted from the inhibition of Na^+-$2Cl^-$-K^+ cotransport system (Burg, 1976; Eveloff *et al.*, 1981; Greger *et al.*, 1983; Warnock & Eveloff, 1982).

There were several reports that the electrolytes reabsorption renal tubules were affected by cyclic nucleotides. The interaction between cAMP and ion transport in TALH was well investigated, and although a few exceptions (Stoner, 1977; Sasaki & Imai, 1980), most investigators reported that cAMP has a role of mediator or modulator of NaCl transport in TALH (Hebert & Andreoli, 1984b; Schlatter & Greger, 1985; Takaichi *et al.*, 1986). However, the role of cGMP in salt reabsorption of TALH is not yet defined. Although Lee and Cho (1983), and Lee *et al.* (1985) reported the relation between the change in renal tissue level of cGMP and the action of furosemide, these were not sufficient to interpret the role of cGMP in salt reabsorption of TALH. In this study using a relatively defined mTALH cell, loop diuretics, furosemide and ethacrynic acid, increased the activity of guanylate cyclase and these effects was not blocked by aspirin and indomethacin. These results suggest that loop diuretics have the stimulatory effect of guanylate cyclase which is not related with prostaglandins. But, arachidonic acid potentiated the effects of furosemide on guanylate cyclase. Therefore furosemide also has a stimulatory effect on guanylate cyclase mediated by prostaglandin. Prostaglandins stimulated significantly the guanylate cyclase, but arachidonic acid, even though the relatively high concentration, revealed the insignificant effect on guanylate cyclae. These results were due to that cyclooxygenase activity, and hence the rate of prostaglandin production, is negligibly small in the mTALH (Smith & Wilkin, 1977; Farman *et al.*, 1987).

The cAMP was proposed as a principal mediator or modulator in controlling of ion transport in mTALH (Hebert and Andreoli, 1984a). But, according to the results that loop diuretics which exert effect on TALH stimulated significantly guanylate cyclase of mTALH, cGMP may be also related to the ion transport system in mTALH.

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= 국문초록 =

흰쥐 헨레고리 수질 비후상행각의 Guanylate Cyclase에 대한 고효능 이뇨제들의 영향

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이석용 · 노경식 · 김옥녀 · 이상복 · 조규철

흰쥐의 헨레고리 수질 비후상행각에서의 전해질 재흡수와 cyclic GMP와의 관계를 알아보고자 수질비후상행각의 guanylate cyclase에 대한 furosemide와 ethacrynic acid의 영향을 관찰하였다. 또한 이들 작용과 prostaglandin의 상관관계를 알아보고자 guanylate cyclase에 대한 고효능이뇨제(furosemide, ethacrynic acid)와 cyclooxygenase 억제제들과의 상호작용을 함께 관찰하였다. furosemide와 ethacrynic acid는 guanylate cyclase의 활성을 현저히 증가시켰으며 이 증가작용은 aspirin이나 indomethacin에 의해 차단되지 않았다. arachidonic acid는 furosemide의 guanylate cyclase 활성증가작용을 유의하게 증강시켰다. 이들의 결과는 furosemide와 ethacrynic acid가 직접적인 guanylate cyclase 활성촉진작용을 가지고 있으며 또한 furosemide는 prostaglandin을 경유한 간접적인 guanylate cyclase 활성촉진작용을 가지고 있음을 나타낸다. 또한 수질 비후상행각에서의 전해질 재흡수에 cyclic GMP가 관여할 가능성을 시사한다.