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Effects of insulin and IGF on growth and functional differentiation in primary cultured rabbit kidney proximal tubule cells

- Effects of IGF-I on Na^+ uptake -

Ho-jae Han, Kwon-moo Park, Jang-hern Lee*, IL-suk Yang*

College of Veterinary Medicine, Hormone Research Center, Chonnam National University
*College of Veterinary Medicine, Seoul National University**

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초대배양된 토끼 신장 근위세뇨관세포의 성장과 기능분화에 대한 insulin과 IGF의 효과
- Na^+ uptake에 대한 IGF-I의 효과 -

한호재 · 박권무 · 이장헌* · 양일석*

전남대학교 수의과대학, 호르몬연구센터
서울대학교 수의과대학 수의생리학교실*

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초 록 : 이온운반계는 생체의 각기 다른 세포의 성장을 조절하는 성장조절인자들의 효과를 매개하는데 깊은 관련이 있는 것으로 보고되고 있다. 신장 근위세뇨관에서 솔변연 Na^+/H^+ 상호운반계는 사구체에서 여과된 나트륨의 재흡수와 수소이온의 분비를 조절하는 중요한 기능을 수행한다. 이 연구는 초대배양된 신장 근위세뇨관세포의 나트륨 운반을 Insulin-like Growth Factor-I(IGF-I)이 어떤 경로를 통하여 조절하는지를 알아보고자 실시하였다. 결과는 아래와 같다.

1. 초대배양된 신장 근위세뇨관세포에서 Na^+ uptake는 시간의존적으로 증가되었으며, 30분동안 Na^+ uptake를 실시한 결과 세포외 NaCl 농도의존적으로 Na^+ uptake를 유의성있게 감소시켰다(대조군; 40.11±1.76, 140mM군; 17.82±0.94 pmole Na^+/mg protein/min).

2. Na^+ uptake는 iodoacetic acid(IAA, 1×10^{-4}M) 또는 valinomycin(5×10^{-6}M)처리시 대조군에 비해 각각 50.51±4.4%와 57.65±2.27% 억제되었으며, ouabain(5×10^{-5}M)을 처리한 경우에는 140.23±3.37% 증가되었다. IGF-I(1×10^{-5}M)으로 배양한 세포를 actinomycin D(1×10^{-7} M)와 cycloheximide(4×10^{-5}M)로 처리시 Na^+ uptake는 대조군에 비해 각각 90.21±2.39%와 89.64±3.69%로 감소되었다.

3. IGF-I으로 배양한 세포에서 세포외 cAMP는 농도의존적(10^{-8}-10^{-4}M)으로 Na^+ uptake를 유의성있게 감소시켰고, 3-isobutyl-1-methyl-xanthine(IBMX, 5×10^{-5}M)도 억제시켰다. Pertussis toxin (PTX, 50pg/ml)이나 cholera toxin(CTX, 1μg/ml)의 처리시에도 Na^+ uptake는 억제되었다. 세포외

Address reprint requests to Dr. Ho-jae Han, College of Veterinary Medicine, Chonnam National University, Kwang-ju 500-757, Republic of Korea.

phorbol 12-myristate 13 acetate(PMA) 또한 농도의존적(1-100ng/ml)으로 Na^+ uptake를 감소시켰다. 그러나 staurosporine(1×10^{-7}M)은 Na^+ uptake에 영향을 미치지 않았으며 PMA와 staurosporine을 동시에 처리했을 때도 Na^+ uptake는 억제되지 않았다.

결론적으로 초대배양된 토끼 신장 근위세뇨관세포에서 Na^+ uptake는 막전위와 세포내 에너지 의존적이며 IGF-I은 부분적으로 단백질 및 RNA 합성을 통해서 그리고 세포내 cAMP나 PKC 경로를 통해서 Na^+ uptake를 조절하는 것으로 생각된다.

Key words : kidney, cell culture, IGF, sodium.

Introduction

Kidney tubule epithelial cells are of particular interest in these regards as *in vivo* these cells are involved in the reabsorption of solutes from the luminal fluid. Such reabsorption in many cases depends upon the Na^+/H^+ antiport system and the Na^+/K^+-ATPase[1]. In the renal proximal tubule, sugars, amino acids and phosphate are reabsorbed. This reabsorption occurs initially via specific cotransport systems, which transport the solute and Na^+ into the proximal tubule cells[2]. Reabsorption via these systems thus depends upon Na^+/K^+-ATPase activity. All of these transport processes, furthermore may be hormonally regulated. In the renal proximal tubule hormone such as insulin has been observed to increase the activity of the Na^+/H^+ antiport system and the Na^+/phosphate cotransport system[3].

Ion transport systems have been proposed to be intimately involved in mediating the affects of growth regulatory factors on the growth of a number of different types of animal cells *in vivo*. In many tissues, the Na^+/H^+ antiporter is stimulated by mitogenic stimuli[4]. While the brush border membrane form of the transporter may subserve similar functions in renal and small intestinal cells, renal transporter is more likely to be involved in transepithelial transport of electrolytes[5,6]. The functional importance of the apical membrane Na^+/H^+ antiporter in the renal proximal tubule is evidenced by estimates that this transporter mediates the reabsorption of approximately one third of the filtered load of sodium and the bulk of the secretion of hydrogen ions. A role for alterations in Na^+ flux as a component of a generalized renal epithelial response to growth factors is an intriguing possibility. In particular, the potential relationship between alterations in Na^+ flux and renal epithelial growth needs to be considered. Although the endogenous growth factors controlling renal epithelial growth are largely unknown, IGF-I has been implicated. Renal hypertrophy is a manifestation of acromegaly, which is characterized by an increase in circulating IGF-I levels[7]. In addition, in response to certain stimuli, such as the loss of renal mass, the mammalian kidney is known to synthesize IGF-I[8]. Moreover, specific receptors for IGF-I have been found in the proximal tubule[9]. These observations support the concept of an autocrine "loop" for IGF-I and a potential role of this loop in compensatory renal growth.

Previous investigations which have been concerned with hormonal control of renal transport processes have primarily used tissue removed directly from the animal. Investigations with such material, however, have certain inherent limitations. Such tissue has a short life span after being removed from the animal and thus is not in a steady state condition. Although this problem is avoided in whole animal studies, hormonal affects on kidney cells are not necessarily being directly examined when using this approach. These problems may be alleviated to a major extent by the use of *in vitro* renal cell culture systems. More precise and reproducible solute uptake studies may be conducted with epithelial monolayers *in vitro,* than with slices. The factors which are involved in the control of epithelial transport systems may be readily identified, due to the availability of hormonally defined, serum free culture medium[10]. The primary rabbit kidney proximal tubule cells exhibit a number of pro-

perties typical of the renal proximal tubule[10,11]. The primary cells have been shown to possess several transport systems typical of the renal proximal tubule, including a Na^+/glucose cotransport system, a Na^+/phosphate cotransport system, and a probenecid sensitive p-aminohippuric acid transport system. Control of amiloride-sensitive $^{22}Na^+$ uptake by insulin and PGE_1 has also been demonstrated. In addition, the primary proximal tubule cells have been shown to possess brush border enzymes and parathyroid hormone (PTH) sensitive cyclic AMP production. The primary cells also grow in glucose free medium indicative of gluconeogenic capacity. Therefore this renal proximal tubule cell culture system is useful for *in vitro* experiments of differentiated functions of renal proximal tubule cells. This study was designed to investigate pathway utilized by IGF-I in regulating sodium transport in primary cultured renal proximal tubule cells.

Materials and Methods

Materials : Male New Zealand white rabbits (1.5-2.0kg) were used for these experiments. Dulbecco's Modified Eagle's Medium (D-MEM), F-12 nutrient mixture and soybean trypsin inhibitor were obtained from Life Technologies (Grand Island, NY). Insulin, transferrin, hydrocortisone, IGF-I, PMA, IAA, valinomycin, amiloride, ouabain, actinomycin D, cycloheximide, cAMP, IBMX, acetylsalicylic acid, staurosporine, CTX, PTX and other chemicals were purchased from Sigma Chemical Corp (St. Louis, MO). Class IV collagenase was supplied by Worthington (Freehold, NJ). Sodium($^{22}Na^+$) was purchased from Dupont/NEN. Liquiscint was obtained from National Diagnostics (Parsippany, NY). All other reagents were of the highest purity commercially available. Iron oxide was prepared by the method of Cook and Pickering[12]. Stock solution of iron oxide in 0.9% NaCl was sterilized using an autoclave and diluted with phosphate buffered saline (PBS) prior to use.

Methods Isolation of rabbit renal proximal tubules and culture conditions : Primary rabbit renal proximal tubule cell cultures were prepared by a modification of the method of Chung *et al*[11]. The basal medium, D-MEM/F12, pH 7.4, was a 50 : 50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12. The basal medium was further supplemented with 15mM HEPES buffer(pH 7.4) and 20mM sodium bicarbonate. Immediately prior to the use of the medium, two growth supplements(transferrin and hydrocortisone) were added. Water utilized in medium preparation was purified by means of a MilliQ deionization system. Kidneys of a male New Zealand white rabbit were perfused via the renal artery, first with PBS, and subsequently with D-MEM/F12 containing 0.5% iron oxide (wt/vol) until the kidney turned grey-black in color. Renal cortical slices were prepared by cutting the renal cortex and then homogenized with 4 strokes of a sterile glass homogenizer. The homogenate was poured first through a 253μm and then a 83μm mesh filter. Tubules and glomeruli on top of the 83μm filter were transferred into sterile D-MEM/F12 medium containing a magnetic stirring bar. Glomeruli (containing iron oxide) were removed with a magnetic stirring bar. The remaining proximal tubules were briefly incubated in D-MEM/F12 containing 80μg/ml collagenase (Class IV) and 0.025% soybean trypsin inhibitor. The dissociated tubules were then washed by centrifugation, resuspended in D-MEM/F12 containing the two supplements, and transfered into tissue culture dishes. Primary rabbit renal proximal tubule cells were maintained at 37℃, in a 5% CO_2-humidified environment in D-MEM/F12 medium, supplemented with 5μg/ml transferrin and 5×10^{-8}M hydrocortisone only or, in addition with IGF-I(5×10^{-10}M)[3]. Medium was changed one day after plating and every two days thereafter.

Na^+ uptake studies : Primary rabbit renal proximal tubule cell cultures were grown to confluence in 35mm dishes containing serum free D-MEM/F12 medium supplemented with 5μg/ml transferrin and 5×10^{-8}M hydrocortisone only or, in addition with IGF-I(5×10^{-10}M). Intact monolayers were then used for uptake studies[3]. For $^{22}Na^+$ uptake studies, the medium was removed by aspiration. The monolayers were washed twice with 100mM Tris-HCl buffer, pH 7.3. Na^+ influx then proceeded at 37℃ for 30 minutes in an uptake buffer(10mM Tris buffer, pH 7.3, made isotonic to 140mM NaCl with choline chloride) containing 0.25μCi/ml $^{22}Na^+$ and 5×10^{-5}M ouabain(pH 7.4). At the end of this

period, the monolayers were gently washed three times with ice-cold 100mM Tris-HCl buffer, pH 7.3. After the final wash, the cells were solubilized in 1ml 0.1% SDS. To determine the $^{22}Na^+$ incorporated intracellularly, nine tenth of each sample was removed and counted in a β-counter (Packard Co.). The remainder of each sample was used for protein determination. The radioactive counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein (i.e., label not removed by washing procedure). All uptake measurements were made in triplicate. Protein determination was performed by a modified Bradford method with bovine serum albumin as standard[13].

Statistical analysis : Results were expressed as means± standard errors(SE). The difference between two mean values was analysed by Student's t-test. The difference was considered statistically significant when $p<0.05$.

Results

Effects of extracellular sodium concentrations on Na^+ uptake : Na^+ uptake into the monolayers was examined using $^{22}Na^+$. To determine the effect of extracellular unlabelled Na^+ concentration on $^{22}Na^+$ uptake, the uptake of $^{22}Na^+$ was studied in the presence of 0, 40, 80 and 140mM of NaCl (Fig 1). Fig 2 depicts $^{22}Na^+$ uptake as function of incubation time in primary cultured cells. The rate of Na^+ uptake into the primary cells was about two times higher in the absence of NaCl (40.11±1.76 pmole Na^+/mg protein/min) than in the presence of 140 mM NaCl(17.82±0.94 pmole Na^+/mg protein/min) at the 30 minute uptake point. Raising the concentration of extracellular NaCl induced a decrease in Na^+ uptake compared with control cells, in a dose dependent manner. These results showed that Na^+ is taken up through a carrier-mediated process in primary cultured cells.

Effects of iodoacetic acid(IAA), amiloride, and valinomycin on Na^+ uptake : The effects of IAA (1×10^{-4}M) on Na^+ uptake is illustrated in Table 1. Na^+ uptake was inhibited to 50.51±4.04% of that of control cells by IAA(a glycolytic inhibitor). Na^+ uptake by the primary proximal tubule cells was significantly increased by ouabain(5×10^{-5}M) treatment(140.23±3.37%)(Fig 3). However, a similar amiloride(1×10^{-5}M) treatment significantly inhibit Na^+ uptake. Valinomycin (5×10^{-6}M) is a K ionophore which has been observed to increase membrane permeability to K^+ ions. As a consequence the membrane potential may be set at a K^+ diffusion potential which is not influenced by the Na^+ concentration in the uptake buffer. Na^+ uptake was inhibited to 57.65±2.27% by valinomycin (Table 1).

Effects of actinomycin D and cycloheximide on IGF-I stimulation of Na^+ uptake : IGF-I stimulated Na^+ transport in proximal tubule cells. To test for an involvement of RNA and/or protein synthesis in the effects of IGF-I(5×10^{-10}M) on the Na^+ transport activity, experiments were performed in the presence of an inhibitor of RNA and/or protein synthesis. Cycloheximide was added to the cells at a concentration of 4×10^{-5}M. This concentration of cycloheximide has been previously shown to block the incorporation of L-[^{35}S]methionine

Table 1. Effects of metabolic inhibitor, potassium and potassium ionophore on Na^+ uptake

Conditions	Na^+uptake(pmole Na^+/mg protein/min)	% of Control uptake
Control(choline chloride 140mM)	40.07±1.97	100.00±4.92
Iodoacetic acid(0.1mM)	20.24±1.62*	50.51±4.04
Valinomycin(5μM)	23.10±0.91*	57.65±2.27
KCl(140mM)	24.86±1.70*	62.04±4.24

Primary cultured proximal tubule cells were incubated in uptake buffer (0.25μci/ml $^{22}Na^+$) containing either iodoacetic acid, valinomycin or 140mM KCl for 30 minutes at 37℃. Uptake determinations were as described in Materials and Methods. Values are the means±S.E. of 9 dishes performed on 3 different experiments(n=9). * $P<0.05$ vs. control.

into total protein within minutes[14]. As shown in Fig 4, cycloheximide inhibited Na^+ uptake in IGF-I treated cells(90.21±2.39% of that of control). Actinomycin D(1×10^{-7}M, a gene transcription inhibitor) also inhibited Na^+ uptake. When actinomycin D and cycloheximide were added together, Na^+ uptake dropped to 91.54±4.41% of that of control in IGF-I treated cells.

Effect of cAMP on IGF-I stimulation of Na^+ uptake : The direct effects of cAMP on Na^+ uptake by primary cultured proximal tubule cells grown in D-MEM/F12 supplemented with transferrin and hydrocortisone only or in addition, with IGF-I were examined. Extracellular cAMP decreased Na^+ uptake in a dose-dependent manner(Fig 5). cAMP concentrations between 10^{-8} and 10^{-4}M significantly inhibited Na^+ uptake. In order to evaluate whether the metabolism of cAMP influenced its action on 68.83±8.44% Na^+ uptake in IGF-I treated cells, the effect of the phosphodiesterase inhibitor IBMX(5×10^{-5}M) was studied. As shown in Fig 6, IBMX inhibited Na^+ uptake compared with a control, most likely as a result of increased intracellular cAMP accumulation. The role of heterotrimeric GTP-binding proteins in signal transduction is well established. They might also be involved in sodium transport. Our results showed that the treatment of IGF-I treated cells with a pertussis toxin(50 pg/ml) known to influence the Gi class of G proteins inhibited Na^+ uptake (64.38±9.28% of that of control). Also the cholera toxin(1μg/ml) which results in the ADP-ribosylation of Gs, which in turn, blocks the intrinsic GTPase activity of the subunit, inhibited Na^+ uptake in IGF-I treated cells(63.67±3.39% of that of control) (Fig 7).

Effect of PMA on IGF-I stimulation of Na^+ uptake : In the physiological state, protein kinase C, involved in signal transduction for various hormones, may regulate cellular functions after being activated by diacylglycerol. This suggests the possible involvement of protein kinase C in Na^+ transport in the proximal tubule cells. The direct effects of PMA on Na^+ uptake by primary cultured proximal tubule cells grown in D-MEM/F12 supplemented with transferrin and hydrocortisone only or in addition with IGF-I were examined. A dose-response relationship for the PMA effect on Na^+ uptake was obtained after 15 hours of treatment with PMA. Extracellular PMA decreased Na^+ uptake in a dose-dependent manner(1-100ng/ml)(Fig 8). 100ng/ml PMA con-

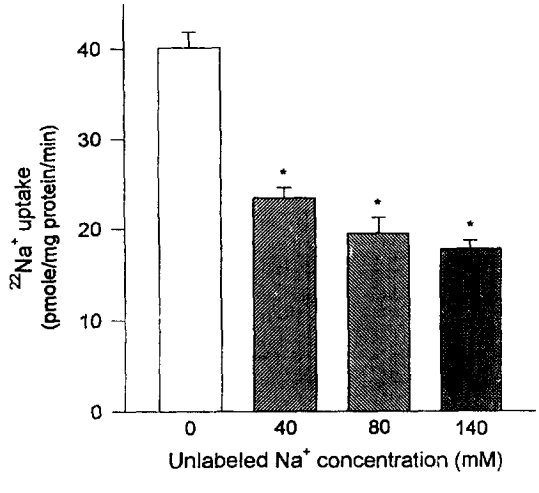

Fig 1. Effects of extracellular sodium concentrations on Na^+ uptake. Primary cultured proximal tubule cells were incubated in uptake buffer(0.25μci/ml $^{22}Na^+$) containing either 0mM (140mM choline chloride), 40mM, 80mM or 140mM NaCl for 30 minutes at 37℃. Values are the means±S.E. of 9 dishes performed on 3 different experiments(n=9).
* P<0.05 vs. 0mM unlabeled Na^+

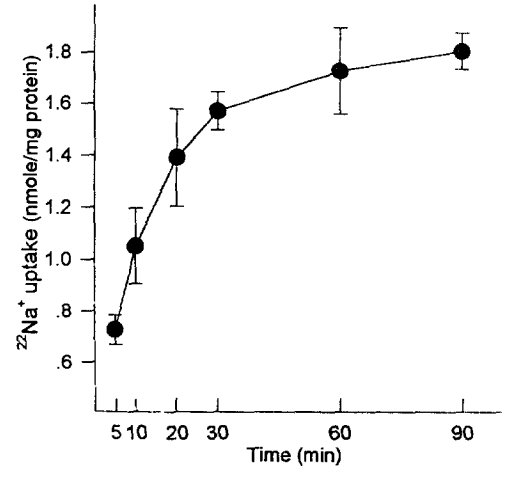

Fig 2. Time course of Na^+ uptake. Na^+ uptake was measured in the presence of 140mM choline chloride. Values are the means±S.E. of 9 dishes performed on 3 different experiments (n=9).

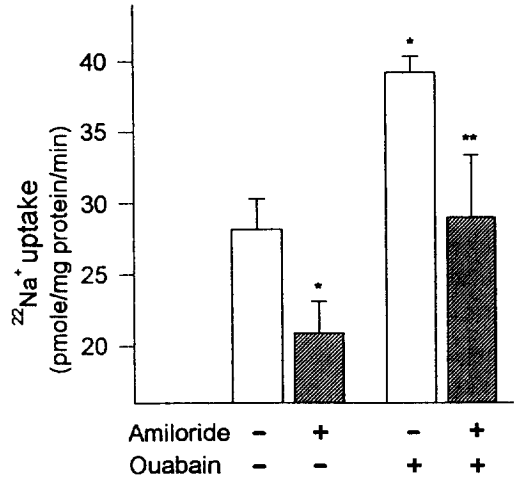

Fig 3. Effects of amiloride and ouabain on Na^+ uptake. Primary cultured proximal tubule cells were preincubated for 3 hours at 37℃ in uptake buffer containing either amiloride(1×10^{-3} M), ouabain(5×10^{-5}M), or both. After the preincubation, the monolayers were incubated in uptake buffer containing 0.25μci/ml $^{22}Na^+$ for 30 minutes. Values are the means±S.E. of 9 dishes performed on 3 different experiments(n=9).
* p<0.05 vs. control, ** p<0.05 vs. ouabain.

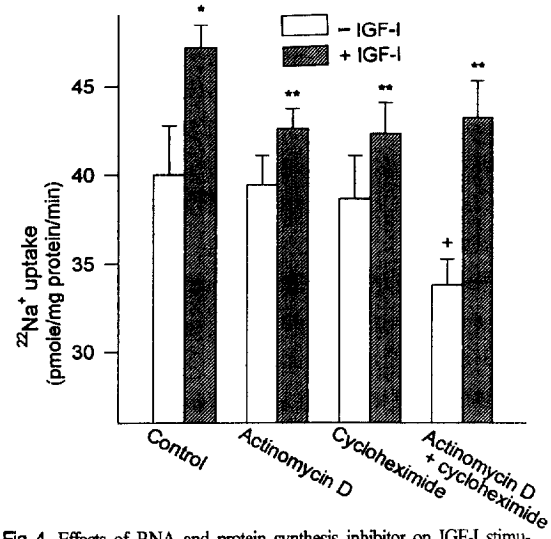

Fig 4. Effects of RNA and protein synthesis inhibitor on IGF-I stimulation of Na^+ uptake. Primary renal proximal tubule cells were grown to confluence in D-MEM/F12 medium supplemented with 5μg/ml transferrin and 5×10^{-8}M hydrocortisone or, in addition with IGF-I (5×10^{-10}M). Then, they were preincubated with actinomycin D(1×10^{-7}M) or cycloheximide(4×10^{-5}M) for 15 hours. After the preincubation, the monolayers were incubated in uptake buffer containing 0.25μci/ml $^{22}Na^+$ for 30 minutes at 37℃. Values are the means±S.E. of 9 dishes performed on 3 different experiments(n=9).
* p<0.05 vs. control in the absence of IGF-I, ** p<0.05 vs. IGF-I alone, + p<0.05 vs. control in the absence of IGF-I.

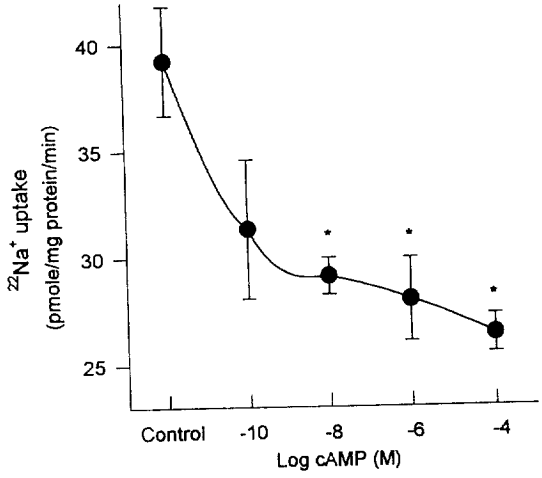

Fig 5. Dose-dependent effect of extracellular cAMP on Na^+ uptake. Primary renal proximal tubule cells were grown to confluence in D-MEM/F12 medium supplemented with 5μg/ml transferrin and 5×10^{-8}M hydrocortisone or, in addition with IGF-I(5×10^{-10}M). Confluent cell monolayers were exposed to cAMP at the given doses for 3 hours prior to determination of Na^+ uptake. After the preincubation, the monolayers were incubated in uptake buffer containing 0.25μci/ml $^{22}Na^+$ for 30 minutes at 37℃. Values are the means±S.E. of 9 dishes performed on 3 different experiments(n=9).
* p<0.05 vs. control.

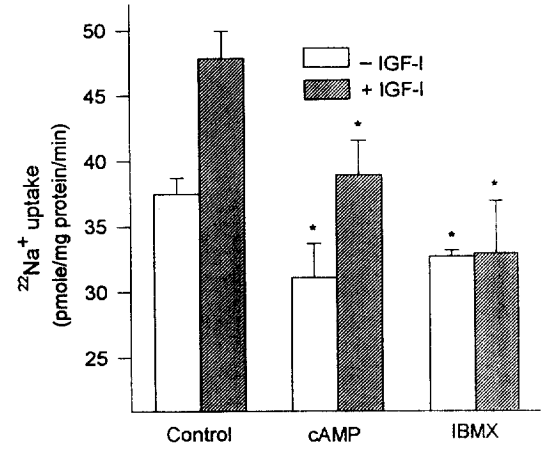

Fig 6. Effects of cAMP on IGF-I stimulation on Na^+ uptake. Primary proximal tubule cells were cultured in D-MEM/F12 medium supplemented with either transferrin (5μg/ml), hydrocortisone (5×10^{-8}M) or, in addition with IGF-I(5×10^{-10}M). The confluent monolayer cells were incubated in the prescence of cAMP(1×10^{-4}M) or IBMX(5×10^{-5}M) at the indicated concentration for 3 hours before uptake. During the uptake period the cells were incubated with uptake buffer, which contain 0.25μci/ml $^{22}Na^+$ and the appropriate agents. Values are the mean± S.E. of 9 dishes performed on 3 different experiments (n=9). * p<0.05 vs. their respective control.

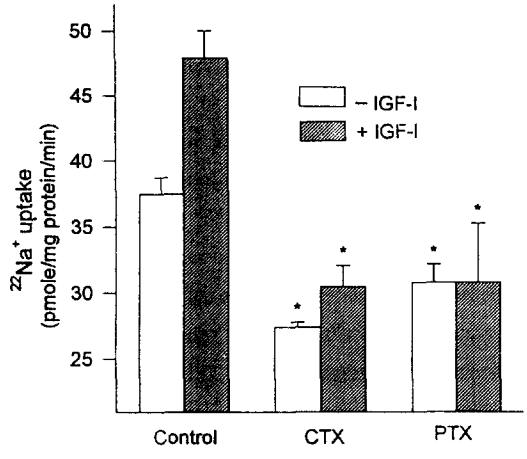

Fig 7. Effects of cholera toxin and pertussis toxin on Na^+ uptake. Primary renal proximal tubule cells were grown to confluence in D-MEM/F12 medium supplemented with 5μg/ml transferrin and 5×10^{-8}M hydrocortisone or, in addition with IGF-I(5×10^{-10}M). Then, they were preincubated with 1μg/ml cholera toxin(CTX) and 50 pg/ml pertussis toxin(PTX) for 3 hours. After the preincubation, the monolayers wee incubated in uptake buffer containing 0.25μci/ml $^{22}Na^+$ for 30 minutes at 37℃. Values are the means±S.E. of 9 dishes performed on 3 different experiments(n=9). * $p<0.05$ vs. their respective control.

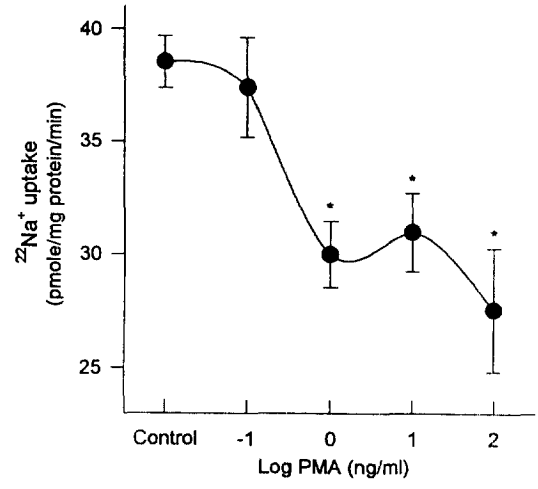

Fig 8. Dose-dependent effect of extracellular PMA on Na^+ uptake. Primary renal proximal tubule cell cultures were grown to confluence in D-MEM/F12 medium supplemented with 5μg/ml transferrin and 5×10^{-8}M hydrocortisone or, in addition with IGF-I(5×10^{-10}M). Confluent cell monolayers were exposed to PMA at the given doses for 3 hours prior to determination of Na^+ transport. After the preincubation, the monolayers were incubated in uptake buffer containing 0.25μci/ml $^{22}Na^+$ for 30 minutes at 37℃. Values are the means±S.E. of 9 dishes per formed on 3 different experiments(n=9).
* $p<0.05$ vs. control.

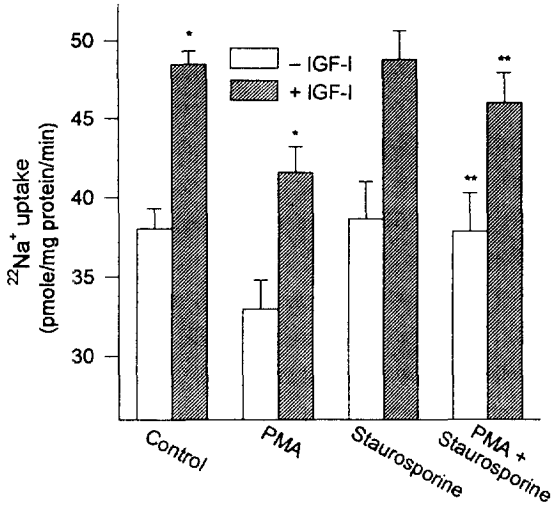

Fig 9. Effects of PMA on IGF-I stimulation of Na^+ uptake. Primary proximal tubule cells were cultured in D-MEM/F12 medium supplemented with either transferrin(5μg/ml), hydrocortisone (5×10^{-8}M) or in addition with IGF-I (5×10^{-10}M). The confluent monolayer cells were incubated in the prescence of any one or combination of PMA (100 ng/ml), staurosporine (1×10^{-7}M) at the indicated concentration for 3 hours before uptake. During the uptake period the cells were incubated with uptake buffer, which contains 0.25μci/ml $^{22}Na^+$ and the appropriate agents. Values are the mean±S.E. of 9 dishes performed on 3 different experiments (n=9). * $p<0.05$ vs. their respective control, ** $p<0.05$ vs. their repective PMA.

centration significantly inhibited Na^+ uptake (85.78 ±3.34% of that of control) in IGF-I treated cells. However, staurosporine (1×10^{-7}M) had no effect on Na^+ uptake. When PMA and staurosporine were added together, inhibition of Na^+ uptake was not observed (Fig 9).

Discussion

The various nephron segments take part in NaCl reabsorption, the primary driving force for which is the Na^+/K^+-ATPase, are localized in the basolateral membrane. The segments have different modalities of Na^+ uptake : by Na^+/H^+ exchange in the proximal tubule, by $Na^+/2Cl^-/K^+$ cotransport in the thick ascending loop, by Na^+/Cl^- cotransport in the distal tubule, and via Na^+ channels in the principal cell of the collecting duct[15]. In the present study, ouabain-treated cells were used for $^{22}Na^+$ uptake, as in the absence of ouabain Na^+ efflux may possibly occur via Na^+/K^+-ATPase, and thus af-

fect determinations of the initial rate of Na^+ uptake. The possibility that multiple Na^+/H^+ antiporter isoform exists was suggested by studies demonstrating differential sensitivity of Na^+/H^+ antiporters to amiloride and amiloride analogs. Proximal tubule cell likely express two type of Na^+/H^+ antiporter[16]. The apical membrane Na^+/H^+ antiporter is encodes by Na^+/H^+ exchanger (NHE-3), while the basolateral membrane Na^+/H^+ antiporter is encoded by NHE-1[17,18]. NHE-3 mRNA expression is high in kidney and higher in kidney cortex than in medulla. NHE-3 encodes an amiloride and ethylisopropylamiloride-resistant Na^+/H^+ antiporter[19,20]. NHE-1 encodes an amiloride-sensitive Na^+/H^+ antiporter that is ubiquitously distributed and likely mediates housekeeping functions such as cell pH regulation, cell growth, cell volume defense, and signal transduction. The present result showed that amiloride partially blocked Na^+ uptake in primary cultured proximal tubule cells. From this result we cannot suggest that what kinds of isoforms exist in primary cultured proximal tubule cells. Additional experiments will be required to resolve this question.

In renal epithelial cells a rise in sodium uptake across the apical membrane increases intracellular sodium concentration, which in turn stimulates the turnover rate of Na^+/K^+-ATPase and thereby enhances sodium efflux across the basolateral membrane[21]. A rise in intracellular sodium concentrations may also stimulate the production of autocrine growth factors that directly or indirectly regulate cell growth and proliferation, by modifying responses to mitogens or to changes in the ionic composition of the extracellular fluid[1]. In the present and in previous studies, $^{22}Na^+$ and $^{86}Rb^+$ uptake were significantly increased in proximal tubule cells incubated with IGF-I[3]. An increase in the glomerular filtration rate and in tubular Na^+ reabsorption from the parental administration of IGF-I have been reported in human subjects. IGF-I levels were higher in hypertensive patients as compared to controls[22]. These results indicated that an association exists between exaggerated circulating levels of IGF-I and abnormalities of renal function present in some patients with essential hypertension. As compared to normotensives and hypertensives with normal IGF-I levels, patients with increased IGF-I levels were characterized by lower fractional Na^+ excretion and higher creatine clearance. In the adult kidney, IGF-I predominates and can influence renal function/morphology in normal and disease conditions, such as compensatory hypertrophy, hypersomatotropism, and diabetes mellitus, and participate in tissue regeneration/recovery of tubular function following acute and chronic injury[23-25].

In order to evaluate whether Na^+ is absorbed into the primary cells by means of a carrier-mediated process, the dependence of the initial rate of Na^+ uptake upon the extracellular Na^+ concentration was evaluated using primary cells incubated with ouabain. The initial rate of Na^+ uptake in ouabain-treated cells exhibited "saturation" with increasing concentrations of extracellular Na^+. This observation would be consistent with the existence of a carrier-mediated process. "Saturation kinetics" may also be affected by (or even be entirely due to) a membrane potential effect. As the extracellular Na^+ concentration is increased, the magnitude of the membrane potential may decrease if the plasma membrane is even slightly permeable to Na^+. As a consequence the initial rate of Na^+ uptake may decline as the extracellular Na^+ concentration is increased. The present result showed that $^{22}Na^+$ uptake was decreased by increased NaCl concentrations or valinomycin treatment in the uptake buffer. This data suggest that Na^+ uptake is indeed sensitive to the membrane potential. The dependence of Na^+ uptake on metabolic energy was also examined. The inhibitory effects of iodoacetate on Na^+ uptake may be explained as a consequence of its effects on intracellular ATP levels. Iodoacetate, a sulfhydryl reagent, would presumably decrease cellular ATP levels as a consequence of its known inhibitory effect on glycolysis.

IGF-I increased Na^+/H^+ antiporter in the luminal brush-border membrane of the proximal tubule. However, the intracellular mechanism is not understood. The stimulatory effect of IGF-I on Na^+ uptake was only partially prevented by cycloheximide, suggesting that both protein synthesis-dependent and -independent mechanisms are involved in the development of the response. The inhibition of Na^+ uptake was also partially dependent on gene transcription because it was slightly inhibited by actinomycin D. These data indicate that IGF-I increases Na^+/H^+ antiporter selectively through a mechanism that involves RNA and/or protein synthesis. These observations suggest that growth and growth hormone-related stimulation of renal Na^+ transport could be

mediated by IGF-I.

The role of extracellular cAMP responsible for the stimulation of the transport of Na^+ in response to IGF-I was examined. Infusion of a permeable analogue of cAMP also resulted in a decrease in the reabsorption of phosphate, sodium, and water in the proximal tubule. Studies in the rat and rabbit established that cAMP inhibited sodium transport in the proximal tubule[26,27]. In LLC-PK_1/PKE_{20} cells, basolateral Na^+/H^+ exchange is more sensitive to amiloride inhibition than is apical Na^+/H^+ exchange. Furthermore, the two exchange activities differ in their regulatory control: Kinase A activation (forskolin, 8-Br-cAMP) leads to inhibition of both exchange activities, whereas kinase C activation (phorbol ester) stimulates basolateral and inhibits apical Na^+/H^+ exchange[28]. While PKA is known to be present in the cytosol of cells, studies in the brush border membrane of dog and rabbit suggest that PKA is also an intrinsic brush border membrane protein[29,30]. Direct proof of the involvement of PKA with subsequent phosphorylation of brush border membrane proteins in regulating Na^+/H^+ exchange activity was provided by Weinman *et al*[31]. This study established that cAMP acting via PKA inhibited the brush border membrane Na^+/H^+ exchange by a process involving the phosphorylation of one or more brush border membrane proteins. The inhibitory effect of both cAMP and the catalytic subunit of cAMP-dependent protein kinase was blocked by the specific thermostable protein inhibitor of the kinase. These results demonstrate that activation of endogenous membrane-bound cAMP-dependent protein kinase or exposure to exogenous catalytic subunit cAMP-dependent protein kinase inhibits the rate of Na^+/H^+ exchange transport in the brush-border membrane of the rabbit kidney[31]. In present study, extracellular cAMP inhibited Na^+ uptake in a dose-dependent manner. IBMX also inhibited Na^+ uptake. These results suggest that IGF-I may act to decrease levels of intracellular cAMP either through inhibition of adenylate cyclase activity or enhancement of phosphodiesterase activity. In the cAMP systems, the step that follows the binding of the ligand to its receptor at the membrane surface, and that initiates the cell response, is the activation of a regulatory protein that binds GTP (G protein)[32]. Cholera toxin activates Gs by adenosine diphosphate (ADP)-ribosylation of specific amino acid residues in the α-subunit, while pertussis toxin blocks the receptor-mediated activation of Gi and Go, also by ADP-ribosylation of the α-subunit. Thus two different toxins produce the same effect, elevated intracellular cAMP levels. This result demonstrates that Na^+ transport in renal proximal tubule cells seem to be regulated by Gs and Gi proteins, respectively.

Application of phorbol esters to brush border membrane vesicles causes an acute increase in Na^+/H^+ antiporter activity. Conversely, application of phorbol esters to cultured cells inhibits amiloride-resistant Na^+/H^+ antiporter activity acutely[33,34]. The acute effect of PKC on the Na^+/H^+ antiporter represents the only discrepancy between regulation of the proximal tubule apical membrane Na^+/H^+ antiporter and regulation of NHE isoforms. The chronic effect may play a role in the increase in Na^+/H^+ antiporter activity seen in chronic acidosis, chronic K^+ deficiency, and chronic hyperfiltration. Tsai and co-workers observed that an active phorbol ester stimulated Na^+/H^+ exchange activity using a primary culture of rabbit renal proximal tubule cells[35]. Baum and Hays reported that activators of PKC inhibited sodium, water, and bicarbonate reabsorption in microperfused superficial rabbit proximal convoluted tubules[36]. In a study using a similar protocol, Wang and Chan observed that phorbol esters stimulated sodium, water, and bicarbonate reabsorption initially[37]. After approximately 15 to 30 minutes of perfusion with phorbol esters, there was a decrease in these measured parameters. The reduction in transport corresponded in time to that observed by Baum and Hays[36]. Thus it appears that activation of PKC does stimulate Na^+/H^+ activity in intact rabbit renal proximal tubules. In addition, phorbol esters rapidly down-regulate the activity of PKC in this nephron segment with a resultant decrease in the activity of the transporter. It is difficult to reconcile these observations with our present results. A number of possible explanations exist for this apparent discrepancy. First, it is possible that the renal proximal tubule provides a unique environment leading to tissue specific regulation of Na^+/H^+ antiporter. Second, it is possible that the decrease in Na/H antiporter activity observed in response to PKC activation involves a different isoform. Lastly, it is pos-

sible that the antiporter is regulated in an aberrant fashion when agonists are applied directly to cultured cells. Additional experiments will be required to resolve these questions. The present results suggest that PKC is one of the possible regulators in transducing the IGF-I signal for sodium transport in primary cultured proximal tubule cells.

In conclusion, sodium uptake in primary cultured rabbit renal proximal tubule cells depends on membrane potentials and intracellular energy levels. IGF-I stimulates sodium uptake through mechanisms that partially involve de novo protein and/or RNA synthesis, and cAMP or PKC pathway.

Summary

It has been suggested that ion transport systems are intimately involved in mediating the effects of growth regulatory factors on the growth of a number of different types of animal cells *in vivo*. The functional importance of the apical membrane Na^+/H^+ antiporter in the renal proximal tubule is evidenced by estimates that this transporter mediates the reabsorption of approximately one third of the filtered load of sodium and the bulk of the secretion of hydrogen ions. This study was designed to investigate the pathway utilized by IGF-I in regulating sodium transport in primary cultured renal proximal tubule cells. Results were as follows :

1. Na^+ was observed to accumulate in the primary cells as a function of time. Raising the concentration of extracellular NaCl induced an decrease in Na^+ uptake compared with control cells in a dose dependent manner. The rate of Na^+ uptake into the primary cells was about two times higher in the absence of NaCl(40.11 ± 1.76 pmole Na^+/mg protein/min) than in the presence of 140mM NaCl(17.82 ± 0.94 pmole Na^+/mg protein/min) at the 30 minute uptake.

2. Na^+ uptake was inhibited by IAA(1×10^{-4}M) or valinomycin(5×10^{-6}M) treatment(50.51 ± 4.04 and 57.65 ± 2.27 of that of control, respectively). Na^+ uptake by the primary proximal tubule cells was significantly increased by ouabain (5×10^{-5}M) treatment(140.23 ± 3.37% of that of control). When actinomycin D(1×10^{-7}M) or cycloheximide(4×10^{-5}M) was applied, Na^+ uptake was decreased to 90.21 ± 2.39% or 89.64 ± 3.69% of control in IGF-I(1×10^{-5}M) treated cells, respectively.

3. Extracellular cAMP decreased Na^+ uptake in a dose-dependent manner(10^{-8}-10^{-4}M). IBMX(5×10^{-5}M) also inhibited Na^+ uptake. Treatment of cells with pertussis toxin(50pg/ml) or cholera toxin(1μg/ml) inhibited Na^+ uptake. Extracellular PMA decreased Na^+ uptake in a dose-dependent manner(1-100ng/ml). 100 ng/ml PMA concentration significantly inhibited Na^+ uptake in IGF-I treated cells. However, staurosporine(1×10^{-7}M) had no effect on Na^+ uptake. When PMA and staurosporine were added together, the inhibition of Na^+ uptake was not observed.

In conclusion, sodium uptake in primary cultured rabbit renal proximal tubule cells was dependent on membrane potentials and intracellular energy levels. IGF-I stimulates sodium uptake through mechanisms that involve some degree of de novo protein and/or RNA synthesis, and cAMP and/or PKC pathway mediating the action mechanisms of IGF-I.

참 고 문 헌

1. Stanton BA, Kaissling B. Regulation of renal ion transport and cell growth by sodium. *Am J Physiol*, 257 : 1-10, 1989.
2. Koushanpour E. Renal physiology, principles and functions. Phil Sounders, 1976.
3. Han HJ, Park KM. Effects of insulin and IGFs on growth and functional differentiation in primary cultured rabbit kidney proximal tubule cells. I. Growth and membrane transport. *Kor J Physiol*, 29 : 191-202, 1996.
4. Wakabayash S, Sardet C, Fafournoux P, *et al.* Structuke and function of the growth factor-activatable Na^+/H^+ exchanger(NHE 1). Rev physiol Biochem pharmacol, 119 : 157-186, 1992.
5. Rabito CA. Sodium cotransport processes in renal epithelial cell lines. *Mineral Electrolyte Metab*, 12 : 32-41, 1986.
6. Saier MH, Boerner P, Grenier FC, *et al.* Sodium entry pathways in renal epithelial cell lines. *Mineral Electrolyte Metab*, 12 : 42-50, 1986.

7. Gershberg H, Heinemann HO, Stump MH. Renal function studies and autopsy report in a patient with gigantism and acromegaly. *J Clin Endo Metab*, 17 : 377-385, 1957.
8. Stiles AD, Sosenko IRS, D'Ercole AJ, *et al.* Relation of kidney tissue somatomedin-C/ insulin-like growth factor I to postnephrectomy renal growth in the rat. *Endocrinol*, 117 : 2,397-2,401, 1985.
9. Hammerman MR, Rogers S. Distribution of IGF receptors in the plasma membrane of proximal tubular cells. *Am J Physiol*, 253 : F841-F847, 1987.
10. Han HJ. Hormonal regulation of the growth and the functional differentiation of renal proximal tubule cells. *Seoul National University phD. Thesis.* 1993.
11. Chung SD, Alavi N, Livingston D, *et al.* Characterization of primary rabbit kidney cultures that express proximal tubule functions in a hormonally defined medium. *J Cell Biol*, 95 : 118-126, 1982.
12. Cook WF, Pickering GW. A rapid method for seperating glomeruli from rabbit kidney. *Nature*, 182 : 1,103-1,104, 1958.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 : 248-254, 1976.
14. Biber J, Murer H. Na-Pi cotransport in LLC-PK_1 cells : fast adaptive response to Pi deprivation. *Am J Physiol*, 249 : C430-C434, 1985.
15. Greger R. The principles of studying ion transport in the kidney tubules. *Fiziologicheskii Zhurnal Imeni I. M. Sechenova*, 80 : 12-21, 1994.
16. Noel J, Pouyssegur. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *Am J Physiol*, 268 : C238-C296, 1995.
17. Orlowski J, Kandasamy RA, Shull GE. Molecular cloning of putative members of the Na/H exchanger gene family. *J Biol Chem*, 267 : 9,331-9,339, 1992.
18. Tse CM, Brant SR, Walker MS, *et al.* Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na/H exchanger isoform(NHE-3). *J Biol Chem*, 267 : 9,340-9,346, 1992.
19. Orlowski J. Heterologous expression and functional properties of amiloride high affinity(NHE-1) and low affinity(NHE-3) isoforms of the rat Na/H exchanger. *J Biol Chem*, 268 : 16,369-16,377, 1993.
20. Tse CM, Levine SA, Yun CHC, *et al.* Functional characteristics of a cloned epithelial Na/H exchanger (NHE3) : Resistance to amiloride and inhibition by protein kinase C. *Proc Natl Acad Sci USA*, 90 : 9,110-9,114, 1993.
21. Lapointe JY, Laprade R, Cardinal J. Characterization of the apical membrane ionic permeability of the rabbit proximal convoluted tubule. *Am J Physiol*, 250 : F339-F347, 1986.
22. Diez J, Ruilope LM, Rodicio JL. Abnormalities of renal function in essential hypertension with increased circulating levels of insulin-like growth factor I. *Archives des Maladies du Coeur et des Vaisseaux*, 85 : 1,189-1,191, 1992.
23. Miller SB, Hansen VA, Hammerman MR. Effects of growth hormone and IGF-I on renal function in rats with normal and reduced renal mass. *Am J Physiol*, 259 : F747-F751, 1990.
24. Werner A, Shenorr Z, Stannard B, *et al.* Experimental diabetes increases insulin-like growth factor I and II receptor concentration and gene expression in kidney. *Diabetes*, 39 : 1,490-1,497, 1990.
25. O'Shea MH, Miller SB, Hammerman MR. Effects of IGF-I on renal function in patients with chronic renal failure. *Am J Physiol*, 264 : F917-F922, 1993.
26. Jacobson HR. Altered permeability in the proximal tubule response to cyclic AMP. *Am J Physiol*, 253 : F71-F79, 1979.
27. Weinman EJ, Benntte SC, Brady RC, *et al.* Effect of cAMP and calmodulin inhibitors on water absorption in rat proximal tubule. *Proc Soc Exp Biol Med*, 176 : 322-326, 1984.
28. Casavola V, Helmle-Kolb C, Murer H. Separate regulatory control of apical and basolateral Na^+/H^+ exchange in renal epithelial cells. *Biochem Biophys Res Comm*, 165 : 833-837, 1989.
29. Hammerman MR. Phosphorylation of type II cAMP-

dependent protein kinase in renal brush border membranes. *Am J Physiol*, 250 : F659-F666, 1986.

30. Shenolikar S, Fischer K, Chang L, *et al.* Type II cAMP-dependent protein kinase is associated with the rabbit kidney brush border membranes. *Adv Sec Messengers Phosphoprotein Res*, 12 : 95-104, 1988.
31. Weinman EJ, Shenolikar S, Kahn AM. cAMP-associated inhibition of Na^+-H^+ exchanger in rabbit kidney brush-border membranes. *Am J Physiol*, 252 : 19-25, 1987.
32. Pimpliker SW, Simons K. Regulation of apical transport in epithelial cells by Gs class of heterotrimeric G protein. *Nature*, 362 : 456-458, 1993.
33. Weinman EJ, Shenolikar S. Protein kinase C activates the renal apical membrane Na/H exchanger. *J Membr Biol*, 93 : 133-139, 1986.
34. Cano A, Preisig PA, Miller RT, *et al.* PTH acutely inhibits and chronically stimulates the Na/H antiporter in OKP cells.(abstract) *JASN*, 4 : 834, 1993.
35. Tsai CJ, Ives HE, Alpern RJ, *et al.* Increased Vmax for Na^+/H^+ antiporter activity in proximal tubule brush border vesicles from rabbits with metabolic acidosis. *Am J Physiol*, 247 : 339-343, 1984.
36. Baum M, Hays SR. Phorbol myristate acetate and dioctanoylglycerol inhibit transport in rabbit proximal convoluted tubule. *Am J Physiol*, 254 : 9-14, 1988.
37. Wang T, Chan YL. Time-and dose-dependent effects of protein kinase C on proximal bicarbonate transport. *J Membr Biol*, 117 : 131-139, 1990.