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Effects of Insulin and IGFs on Phosphate Uptake in Primary Cultured Rabbit Renal Proximal Tubule Cells

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=ABSTRACT=

The aim of present study was to characterize phosphate uptake and to investigate the mechanism for the insulin and insulin-like growth factor(IGF) stimulation of phosphate uptake in primary cultured rabbit renal proximal tubule cells. Results were as follows :

1. The primary cultured proximal tubule cells had accumulated 6.68±0.70 nmole phosphate/mg protein in the presence of 140 mM NaCl and 2.07±0.17 nmole phosphate/mg protein in the presence of 140 mM KCl during a 60 minute uptake period. Raising the concentration of extracellular phosphate to 100 mM(48.33±1.76 pmole/mg protein/min) induced decrease in phosphate uptake compared with that in control cells maintained in 1 mM phosphate(190.66±13.01 pmole/mg protein/min). Optimal phosphate uptake was observed at pH 6.5 in the presence of 140 mM NaCl. Phosphate uptake at pH 7.2 and pH 7.9 decreased to 83.06±5.75% and 74.61±3.29% of that of pH 6.5, respectively.

2. Phosphate uptake was inhibited by iodoacetic acid(IAA) or valinomycin treatment (62.41± 4.40% and 12.80±1.64% of that of control, respectively). When IAA and valinomycin were added together, phosphate uptake was inhibited to 8.04±0.61% of that of control. Phosphate uptake by the primary proximal tubule cells was significantly reduced by ouabain treatment(80.27±6.96% of that of control). Inhibition of protein and/or RNA synthesis by either cycloheximide or actinomycin D markedly attenuated phosphate uptake.

3. Extracellular cAMP and phorbol 12-myristate 13 acetate(PMA) decreased phosphate uptake in a dose-dependent manner in all experimental conditions. Treatment of cells with pertussis toxin or cholera toxin inhibited phosphate uptake. cAMP concentration between 10^{-6} M and 10^{-4} M significantly inhibited phosphate uptake. Phosphate uptake was blocked to about 25% of that of control at 100 ng/ml PMA. 3-Isobutyl-1-methyl-xanthine(IBMX) inhibited phosphate uptake. However, in the presence of IBMX, the inhibitory effect of exogenous cAMP was not significantly potentiated. Forskolin decreased phosphate transport. Acetylsalicylic acid did not inhibit phosphate uptake. The 1,2-dioctanoyl-sn-glycorol(DAG) and 1-oleoyl-2-acetyl-sn- glycerol(OAG) showed a inhibitory effect. However, staurosporine had no effect on phosphate uptake. When PMA and staurosporine were treated together, inhibition of phosphate uptake was not observed.

In conclusion, phosphate uptake is stimulated by high sodium and low phosphate and pH 6.5 in the culture medium. Membrane potential and intracellular energy levels are also an important factor

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for phosphate transport. Insulin and IGF-I stimulate phosphate uptake through a mechanisms that involve de novo protein and/or RNA synthesis and decrease of intracellular cAMP level. Also protein kinase C(PKC) is may play a regulatory role in transducing the insulin and IGF-I signal for *phosphate transport in primary cultured proximal tubule cells.*

Key Words: Kidney, Insulin, IGF, Phosphate

INTRODUCTION

The solute excretion and reabsorption are carried out by various membrane transporters of kidney, such as Na/glucose cotransport, Na/H antiport, Na/phosphate cotransport and Na,K-ATPase. Inorganic phosphate plays an important role in the functions of organic molecules and in the regulation of cellular metabolism. It is also an essential determinant of cellular growth and replication, being a limiting factor in the stimulation of DNA synthesis by mitogenic agents (Becker & Rozengurt, 1982). Na/phosphate cotransport systems of the brush-border membrane in proximal tubules play a major role in reabsorption of phosphate and maintenance of phosphate homeostasis (Murer, 1992). In addition, their activities are thought to be important for the maintenance of metabolic functions in the proximal tubular cells (Hammerman, 1986). Reabsorption of phosphate in nephron segment is altered by various hormones, drugs and dietary phosphorus intake (Bonjour & Caverzasio, 1984). In physiological conditions, parathyroid hormone, glucocorticoids, vitamin, calcium and other serum components act as regulators of phosphate reabsorption by proximal tubule cells (Hammerman, 1986). In most of these situations, the change in phosphate reabsorption is accompanied by a specific change in the capacity of the Na/phosphate cotransport system in brush border membrane.

Growth hormone increases renal phosphate reabsorption in hypophysectomized rats (Caverzasio et al, 1990), isolated perfused proximal tubules (Quigley & Baum, 1991) and opossum kidney (OK) cells (Caverzasio & Bonjour, 1989). It is likely that the effect of growth hormone on phosphate transport is mediated by IGF-I. IGF-I affected phospholipid metabolism. IGF-I increased the level of phosphodiesters more than that of phosphomonoesters. This is consistent with a larger increment in degradation than in synthesis of phospholipids (Roberts & Dennis, 1989). Changes in metabolism of phospholipids may alter membrane composition, membrane protein-lipid interactions, lipid traffic, and the concentration of lipid-derived second messengers (Bonventre & Nemenoff, 1991). Any one or a combination of these factors can affect Na/phosphate contransport. This information suggests that IGF-I could play a critical role in control of the renal transport of phosphate in relation to growth (Han & Park, 1995). The major site of renal synthesis of IGF-I is located at the collecting duct where growth hormone enhances IGF-I gene expression. Collecting ducts do not have receptors for IGF-I (Rogers et al, 1990). Therefore, IGF-I action must be exerted at other sites where IGF-I receptors are present, such as mesangial cells (Aron et al, 1989), and proximal tubular cells (Hammerman & Rogers, 1987). Hirschberg et al (1993) demonstrated that in human subjects, recombinant human IGF-I administration stimulated renal plasma flow and the glomerular filtration rate, increased microproteinuria and tubular phosphate reabsorption, but caused no change in calcium reabsorption. The cellular systems that mediates this hormonal response remains to be investigated.

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. The primary proximal tubule cells have a unique advantage for this study in that they retain hormone responses typical of the renal proximal tubules. Thus renal proximal tubule cell culture system using hormonally defined medium can be used for in vitro studies concerning the mechanism by which hormones and nutrients control the growth and differentiated functions of renal proximal tubule cells (Han et al, 1996) The aim of present study was to characterize phosphate uptake and to investigate the mechanism for the insulin and IGFs stimulation of phosphate uptake in primary cultured rabbit renal proximal tubule cells.

MATERIALS AND METHODS

Materials

Male New Zealand white rabbits (1.5~2.0 kg) 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, phorbol 12-myristate 13 acetate (PMA), 1,2-dioctanoyl-sn-glycorol (DAG), 1-oleoyl-2-acetyl-sn-glycerol (OAG), iodoacetic acid (IAA), valinomycin, amiloride, phloridizin, ouabain, actinomycin D, cycloheximide, 8-bromoadenosine 3':5'-cyclic monophosphate (cAMP), 3-isobutyl-1-methyl-xanthine (IBMX), staurosporine, forskolin, chorela toxin (CTX), pertussis toxin (PTX) and other chemicals were purchased from Sigma Chemical Corp (St. Louis, MO). Class IV collagenase was supplied by Worthington (Freehold, NJ). Phosphate (^{32}P) 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 (1958). Stock solutions of iron oxide in 0.9% NaCl were 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 (1982). 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 15 mM HEPES buffer (pH 7.4) and 20 mM sodium bicarbonate. Immediately prior to the use of 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 253 μm and then a 83 μm mesh filter. Tubules and glomeruli on top of the 83 μm 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 80 μg/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°C, in a 5% CO_2-humidified environment in D-MEM/F12 medium, supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone only or, in addition, with either insulin (5×10^{-10} M), IGF-I(5×10^{-10} M), or IGF-II (5×10^{-10}

M) (Chung et al, 1982). Medium was changed one day after plating and every two days thereafter.

Phosphate uptake studies

Primary rabbit renal proximal tubule cell cultures were grown to confluence in 35 mm dishes containing serum free D-MEM/F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone only or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M). Intact monolayers were then used for uptake studies (Han & Park, 1995).

To study phosphate uptake, the culture medium was first removed by aspiration. The monolayers were gently washed twice with the uptake buffer. The uptake buffer contained 150 mM NaCl, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 10 mM MES/Tris, pH 6.5. After the washing procedure, the monolayers were incubated at 37°C for 30 minutes in an uptake buffer that contained 0.5 μCi/ml [^{32}P]-phosphate and 1 mM phosphate. At the end of this incubation period, the monolayers were again washed three times with ice cold uptake buffer. After the final wash, the cells were solubilized in 1 ml 0.1% SDS. To determine the ^{32}phosphate incorporated intracellularly, nine tenth of each sample was removed and counted in a scintillation 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 (1976) with bovine serum albumin as standard.

Statistical analysis

Results were expressed as means±standard errors (S.E.). 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 ions and pH on phosphate uptake

Phosphate uptake into the monolayers was examined using ^{32}phosphate. The uptake of ^{32}P labelled phosphate was studied in the presence of 140 mM NaCl or 140 mM KCl. Phosphate was observed to accumulate into the primary cells as a function of time. The initial rate of phosphate uptake into the primary cells was about threefold higher (189.23± 6.73 pmole phosphate/mg protein/min) in the presence of 140 mM NaCl than in the presence of 140 mM KCl (59.07±4.90 pmole phosphate/mg

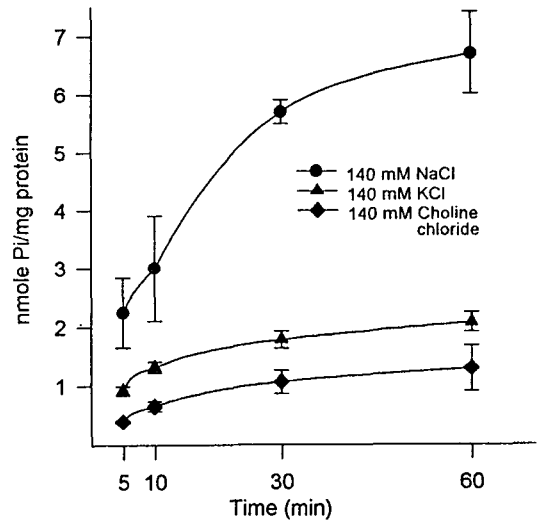

Fig. 1. Time course of phosphate uptake. Phosphate uptake was measured at 1 mM phosphate concentration in the presence of 140 mM NaCl, 140 mM KCl or 140 mM Choline chloride. Uptake was allowed to proceed at 37°C over 60 minute time interval. During this time, the phosphate uptake (0.5 μci/ml ^{32}phosphate) reaction was stopped periodically, and the phosphate incorporated intracellularly was then determined in triplicate dishes. Each point is the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9).

protein/min) at the 30 minute uptake. After a 60 minute uptake period the primary cultured proximal tubule cells had a accumulated 6.68±0.70 nmole phosphate/mg protein in the presence of 140 mM NaCl and 2.07±0.17 nmole phosphate/mg protein in the presence of 140 mM KCl (Fig. 1). Raising the concentration of extracellular phosphate to 100 mM (48.33±1.76 pmole phosphate/mg protein/min) induced an decrease in Na-dependent phosphate uptake compared with that in control cells maintained in 1 mM phosphate (190.66±13.01 pmole phosphate/mg protein/min) (Table 1). This results showed that the characteristics of phosphate in our model system resemble the properties of phosphate transport in the kidney. To examine pH dependency of phosphate uptake, the uptake was compared at pH range of 6.5 to 7.9. As shown in Fig. 2, optimal phosphate uptake was observed at pH 6.5 in the presence of 140 mM NaCl. Phosphate uptake at pH 7.2 and pH 7.9 decreased to 83.06± 5.75% and 74.61±3.29% of that of pH 6.5, respectively.

Table 1. Comparison between low and high extracellular phosphate concentration on phosphate uptake

Conditions	Phosphate uptake (pmole Pi/mg protein/min)	% of uptake at 1mM
1 mM	190.66±13.01	100.00±6.82
100 mM	48.33±1.76*	25.35±1.94

Primary cultured proximal tubule cells were incubated in uptake buffer (0.5 μci/ml ^{32}phosphate) containing 1 mM or 100 mM phosphate for 30 minutes. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9).
* P<0.05 vs. 1 mM.

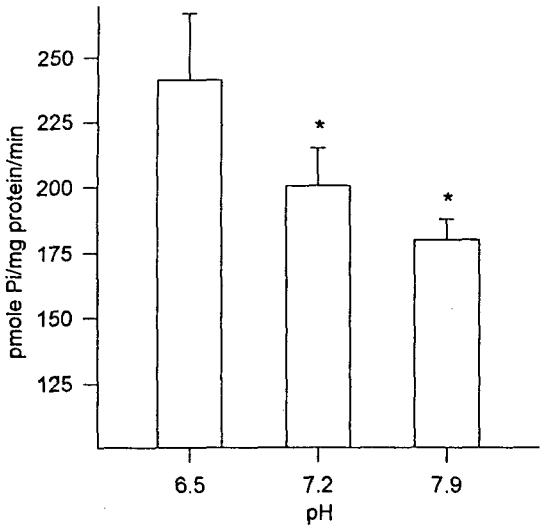

*Fig. 2. Effect of pH on phosphate uptake. Primary cultured proximal tubule cells were incubated with uptake buffer containing 0.5 μci/ml ^{32}phosphate for 30 minutes at 37°C. The pH of uptake buffer was varied at pH 6.5, 7.2 or 7.9, using Tris buffer. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs pH 6.5.*

Table 2. Effects of metabolic inhibitor and potassium ionophore on phosphate uptake

Conditions	Phosphate uptake (pmole Pi/mg protein/min)	% of control uptake
Control	190.66±13.01	100.00±6.82
Iodoacetic acid(0.1 mM)	119.0±8.39*	62.41±4.40
Valinomycin(5 μM)	24.40±3.12*	12.80±1.64
Iodoacetic acid + Valinomycin	15.32±1.17*	8.04±0.61

Primary cultured proximal tubule cells were incubated in uptake buffer (0.5 μci/ml ^{32}phosphate) containing either iodoacetic acid, valinomycin or iodoacetic acid and valinomycin for 30 minutes at 37°C. Values are the means±S.E for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs. control.

Effects of IAA, valinomycin and transporter blockers on phosphate uptake

In order to evaluate whether phosphate uptake depends on membrane potential and intracellular energy level, experiments were conducted. The effects of IAA (1×10^{-4} M) on phosphate uptake is illustrated in Table 2. Phosphate uptake was inhibited to $62.41\pm4.40\%$ of that of control by IAA (aglycolytic inhibitor). Valinomycin (5×10^{-6} M) is a K ionophore which has been observed to increase the membrane permeability to K ions. As a consequence the membrane potential may be rendered a K^+ diffusion potential which is not influenced by the Na^+ concentration in the uptake buffer. Phosphate uptake was inhibited to $12.80\pm1.64\%$ of that of control by valinomycin. When IAA and valinomycin were added together, phosphate uptake was inhibited to $8.04\pm0.61\%$ of that of control. Phosphate uptake by the primary proximal tubule cells was significantly reduced by ouabain treatment ($80.27\pm6.96\%$ of that of control). However, phloridizin or amiloride treatments were not significantly inhibitory to phosphate uptake(Fig. 3).

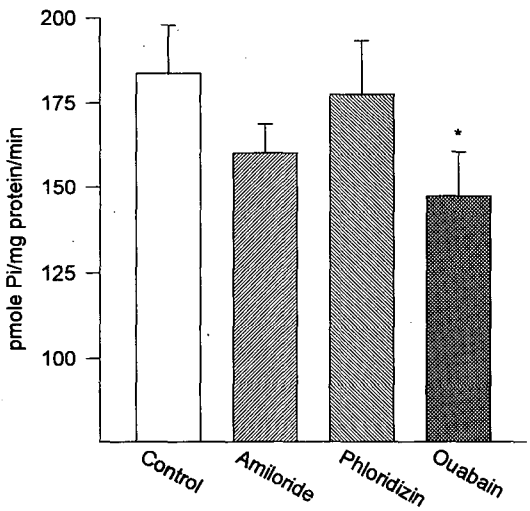

*Fig. 3. Effects of Na^+-dependent transporter blockers on phosphate uptake. Primary cultured proximal tubule cells were preincubated for 3 hours at 37°C in medium containing appropriate blockers. The blockers used included amiloride (1×10^{-5} M), phloridizin (1×10^{-5} M) or ouabain (1×10^{-5} M). After preincubation, the monolayers were incubated in uptake buffer containing 0.5 μci/ml ^{32}phosphate for 30 minutes. The effect of each blocker was examined in triplicate dishes and compared with the control. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs. control.*

Effects of actinomycin D and cycloheximide on insulin and IGF stimulation of phosphate uptake

Insulin and IGF-I stimulated Na/phosphate cotransport in proximal tubule cells. To test for an involvement of RNA and/or protein synthesis in the effects of insulin (5×10^{-10} M), IGF-I (5×10^{-10} M) and IGF-II (5×10^{-10} M) on the Na/phosphate cotransport 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 was shown previously to block the incorporation of L-[^{35}S]methionine into total protein within minutes (Biber & Murer, 1985). As shown in Fig. 4, cycloheximide inhibited phosphate uptake in all experimental conditions. Actinomycin D (1×10^{-7} M, inhibit gene transcription) also inhibited phosphate uptake in insulin or IGF-I treated conditions. When actinomycin D and cycloheximide were added together, phosphate uptake was inhibited in all experimental conditions.

Effect of cAMP on insulin and IGF stimulation of phosphate uptake

cAMP are known to influence renal phosphate handling in mammalian kidneys. The direct effects of cAMP on phosphate uptake by primary cultured proximal tubule cells grown in D-MEM/F12 medium supplemented with transferrin and hydrocortisone only or in addition, with either insulin, IGF-I or IGF-II were examined. Extracellular cAMP

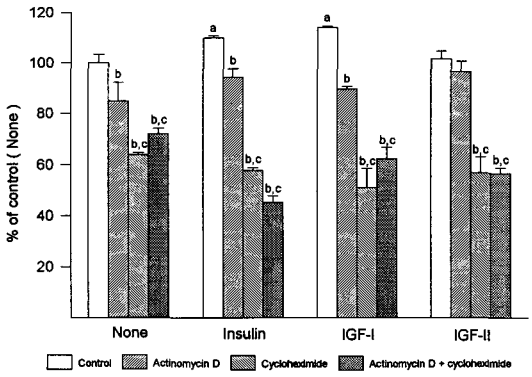

Fig. 4. Effects of RNA and protein synthesis inhibitor on insulin and IGF stimulation of phosphate uptake. Primary renal proximal tubule cells were grown to confluence in D-MEM/F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone (none) or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M) or IGF-II (5×10^{-10} M). Confluent cell monolayers were further incubated in insulin (or IGFs) treated media for 4 days. Then, they were preincubated with either actinomycin D (1×10^{-7} M), cycloheximide (4×10^{-5} M) or actinomycin D and cycloheximide for 15 hours. After the preincubation, the monolayers were incubated in uptake buffer containing 0.5 μci/ml ^{32}phosphate for 30 minutes at 37°C. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n = 9). a P<0.05 vs. control of none; b P<0.05 vs control of each group; c P<0.05 vs. actinomycin D treated condition of each group.

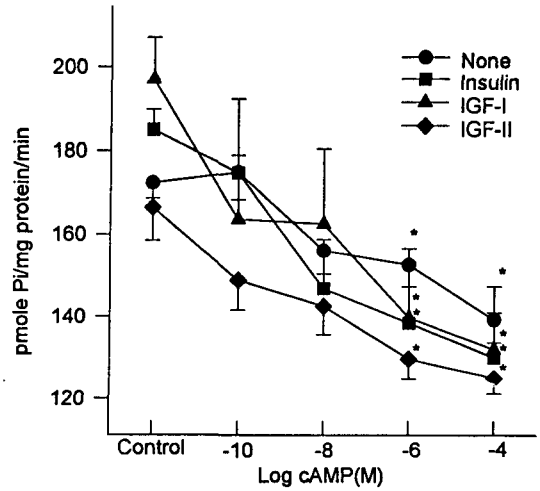

*Fig. 5. Effect of extracellular cAMP on insulin and IGF stimulation of phosphate uptake. Primary renal proximal tubule cells were grown to confluence in D-MEM/F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone (none) or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M) or IGF-II (5×10^{-10} M). Confluent cell monolayers were further incubated in insulin (or IGFs) treated media for 4 days and were subsequently exposed to cAMP at the given doses for 15 hours prior to determination of phosphate uptake. After preincubation, the monolayers were incubated in uptake buffer containing 0.5 μci/ml ^{32}phosphate for 30 minutes at 37°C. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs. control of each group.*

decreased phosphate uptake in a dose-dependent manner in all experimental conditions. cAMP concentration between 10^{-6} and 10^{-4} M significantly inhibited phosphate uptake (Fig. 5). Treatment of cells with pertussis toxin or cholera toxin also inhibited phosphate uptake (Fig. 6). In order to evaluate whether metabolism of cAMP influenced its action on phosphate uptake in insulin treated cells, the effect of the phosphodiesterase inhibitor IBMX (5×10^{-5} M) was studied. As shown in Table 3, IBMX inhibited phosphate uptake (86.36±1.67% of that of control), most likely as result of increased intracellular cAMP accumulation. However, in the presence of IBMX, the inhibitory effect of exogenous cAMP was not significantly potentiated. The effect of activator of the adenylate cyclase-cAMP system on phosphate uptake was also studied. Forskolin decreased phosphate uptake (62.63± 4.56% of that of control), as a result of increased cAMP formation. Acetylsalicylic acid, inhibitor of cAMP formation, does not inhibited phosphate uptake. In the presence of acetylsalicylic acid, the inhibitory effect of cAMP were not observed.

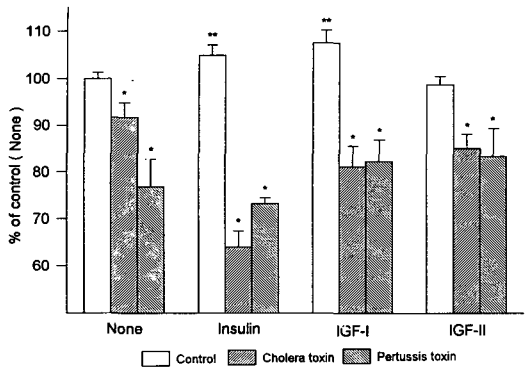

Fig. 6. Effects of cholera toxin and pertussis toxin on phosphate uptake. Primary renal proximal tubule cells were grown to confluence in D-MEM/F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone (none) or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M) or IGF-II (5×10^{-10} M). Confluent cell monolayers were further incubated in insulin (or IGFs) treated media for 4 days. Then, they were preincubated with 1 μg/ml cholera toxin (CTX) and 50 pg/ml pertussis toxin (PTX) for 3 hours. After preincubation, the monolayers were incubated in uptake buffer containing 0.5 μci/ml ^{32}phosphate for 30 minutes at 37°C. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9). a P<0.05 vs. control of none; b P<0.05 vs. control of each group.

Effect of PMA on insulin and IGF stimulation of phosphate 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 phosphate transport in the proximal tubule cells. The direct effects of PMA on phosphate uptake by primary cultured proximal tubule cells grown in D-MEM/F12 medium supplemented with transferrin and hydrocortisone only or in addition, with either insulin, IGF-I or IGF-II were examined. A dose-response relationship for the PMA effect on phosphate uptake was obtained after 15 hours of treatment with PMA. Extracellular PMA decreased phosphate uptake in a dose-dependent manner in all experimental conditions. 100 ng/ml PMA concentration significantly inhibited phosphate uptake (Fig. 7).

Table 4 demonstrates the effect of different PKC activators and PKC inhibitor with respect to

Table 3. Effects of cAMP on insulin-induced stimulation of phosphate uptake

Conditions	Phosphate uptake (pmole Pi/mg protein/min)	% of control uptake
None	168.48±2.57	88.38±1.35
Control(5×10^{-5} M)	190.64±5.63*	100.00±2.95
cAMP(1×10^{-4} M)	170.83±10.02**	89.61±5.26
Forskolin(1×10^{-5} M)	119.40±8.70**	62.63±4.56
IBMX(5×10^{-5} M)	164.63±3.18**	86.36±1.67
Acetylsalicylic acid(1×10^{-3} M)	187.90±10.90	98.56±5.72
IBMX+cAMP	157.37±7.62**	82.55±4.00
Acetylsalicylic acid+cAMP	187.74±6.48	98.48±3.40

Primary proximal tubule cells were cultured in D-MEM/F12 medium supplemented with either transferrin (5 μg/ml), hydrocortisoen(5×10^{-8} M)(none) or in addition, with insulin(5×10^{-10} M)(control). The confluent monolayer cells were incubated in the presence of insulin and any one or combination of these agents at the indicated concentration for 3 hours before uptake. During the uptake period the cells were incubated with uptake buffer, which contain 1 mM phosphate, 0.5 μci/ml ^{32} phosphate, and the appropriate agents. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs. none; ** P<0.05 vs. control.

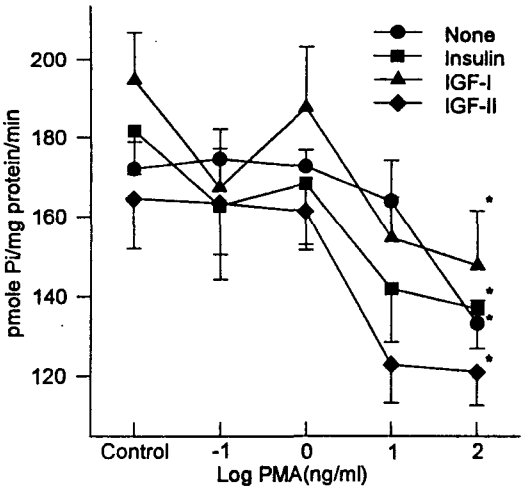

*Fig. 7. Effect of extracellular PMA on insulin and IGF stimulation of phosphate uptake. Primary renal proximal tubule cell cultures were grown to confluence in D-MEM/F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone (none) or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M) or IGF-II (5×10^{-10} M). Confluent cell monolayers were further incubated in insulin (or IGFs) treated media for 4 days and were subsequently exposed to PMA at the given doses for 15 hours prior to determination of phosphate transport. After preincubation, the monolayers were incubated in uptake buffer containing 0.5 μci/ml ^{32}phosphate for 30 minutes at 37°C. Values are the means±S.E. for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs. control of each group.*

phosphate uptake. When primary cultured proximal tubule cells were preincubated with PMA, 2-dioctanoyl-sn-glycorol (DAG), 1-oleoyl-2- acetyl-sn-glycerol (OAG), and staurosporine for 3 hours before phosphate uptake experiments, PMA, DAG and OAG showed a inhibitory effect (93.43±2.03%, 73.33±8.13%, 66.20±5.32% of control, respectively). However, staurosporine had no effect on phosphate uptake. When PMA and staurosporine were added together, inhibition of phosphate uptake was not observed.

DISCUSSION

The transport of phosphate across plasma membrane of the renal proximal tubular epithelial cell is thought to take place through the activities of specific transporters located in the membrane. The initial rate of phosphate uptake into the primary cells was higher in the presence of 140 mM NaCl than in the presence of 140 mM KCl. In the presence of an Na^+ gradient, phosphate is rapidly accumulated in the cells. In the presence of a K^+ gradient, only a slow equilibrating uptake is observed. This experiments in part demonstrate Na^+-dependent phosphate cotransport in primary cultured rabbit proximal tubule cells. Similarly, the measurements of phosphate dependent uptake of $^{22}Na^+$ have shown that Na^+ enters the vesicles together with phosphate, i.e., Na^+ and phosphate are cotransported, with the Na^+ gradient providing the driving force for concentrative uptake of phosphate (Hoffmann et al, 1976). The present results showed that phosphate uptake in primary cells was decreased by raising phosphate concentrations in the culture medium. Lowering the concentration of extracellular phosphate (3.0 mmole/L to 0.3 mmole/L) induced an increase in Na^+-dependent phosphate uptake compared with that in control cell maintained in 3.0 mmole/L phosphate in JTC-12 (Takuwa et al, 1986). Phosphate deprivation (exposure to phosphate-free medium) increased Na^+-dependent phosphate uptake by 1.8- to 5.8- folds and decreased cell inorganic phosphate uptake and ATP contents (70-80 and 17-30%, respectively)(Escoubet et al, 1989, Ernest et al, 1995)

The pH dependence of phosphate uptake was examined. Phosphate uptake by the primary cells occurred at an optimal rate at pH 6.5. The pH optimum for phosphate uptake has been determined similarly in several other renal systems. The electroneutral $2Na^+$ - HPO_4^{2-} cotransport system seems to be dominant, at least in rat proximal tubule

Table 4. Effects of PMA on insulin stimulation of phosphate uptake

Conditions	Phosphate uptake (pmole Pi/mg protein/min)	% of control uptake
None	168.48±2.57	88.38±1.35
Control(5×10^{-5} M)	190.64±5.63*	100.00±2.95
PMA(100 ng/ml)	178.12±3.87**	93.43±2.03
DAG(5×10^{-5} M)	139.80±15.50**	73.33±8.13
OAG(5×10^{-5} M)	126.20±10.15**	66.20±5.32
Staurosporine(1×10^{-7} M)	181.21±1.37	95.05±0.72
Staurosporine + PMA	192.87±3.53	101.70±1.85

Primary proximal tubule cells were cultured in D-MEM/F12 medium supplemented with either transferrin (5 μg/ml), hydrocortisoen (5×10^{-8} M)(none) or in addition, with insulin (5×10^{-10} M)(control). The confluent monolayer cells were incubated in the presence of insulin and any one or combination of these agents at the indicated concentration for 3 hours before uptake. During the uptake period the cells were incubated with uptake buffer, which contain 1 mM phosphate, 0.5 μci/ml ^{32} phosphate, and the appropriate agents. Values are the means±S.E for 9 separate experiments performed on 3 different cultures (n=9). * P<0.05 vs. none; ** P<0.05 vs. control.

(Gmaj & Murer, 1986). The electrogenic component can only be demonstrated at pH values lower than those normally encountered in the proximal tubule, under conditions when the net phosphate transport is very low. Hence, it is not likely that this component plays a major role in renal handling of phosphate. However, the driving force for electrogenic $2Na^+$ - $H_2PO_4^-$ cotransport involves the cell potential in addition to the electrochemical Na^+ gradient. This might be important when tubular [Pi] or tubular pH is very low, e.g., in the late proximal tubule. In rabbit renal brush border membrane vesicles, optimal phosphate uptake was observed at pH values above 7, where the divalent species of phosphate is predominant (Sacktor and Cheng, 1981). The rate of phosphate uptake into intact LLC-PK_1 cells did not change significantly within a pH range of 6.6 to 7.4 (Biber et al, 1983). At pH 6.5 the ratio between monovalent and divalent phosphate is 2 : 1 (the pKa is 6.8). Thus, monovalent phosphate may conceivably be transported over divalent phosphate by the phosphate transport system in primary proximal tubule cells. However, the form of phosphate that is transported cannot be simply determined by determining the pH optimum for phosphate. The present results suggest the existence of a heterogeneity of Na/phosphate cotransport systems within renal tubules in as much as they may differ depending on species.

The membrane potential affects both the affinity for phosphate and the maximal velocity (Vmax) of the transporter. This report indicate that the electrical potential is an important driving force for the Na^+-phosphate carrier and that the translocation of the carrier is a potential-dependent step (Beliveau & Strevey, 1991). The present results showed that phosphate uptake of the primary proximal tubule cells was significantly reduced by ouabain or valinomycin treatment. Ouabain treatment would presumably cause the intracellular Na^+ levels to be elevated, dissipating the Na^+ concentration gradient across the plasma membrane. These observations do not exclude the possibility that phosphate uptake by the primary cells is sensitive to the Na^+ gradient across the plasma membrane or membrane potential. The dependence of phosphate uptake on metabolic energy was also examined. The inhibitory effects of iodoacetate on phosphate uptake may be explained as being a consequence of their effects on intracellular ATP levels. Iodoacetate, a sulfhydryl

reagent, would presumably decrease cellular ATP levels a consequence of its known inhibitory effect on glycolysis. These inhibitory effects were more potentiated when valinomycin and iodoacetate were added together. This result suggests that phosphate uptake depends on mebrane potential and intracellular ATP levels. However, Waqar et al (1985) reported that iodoacetate or ouabain did not significantly inhibit phosphate uptake when added individually to the uptake buffer or slightly inhibitory effect (i.e., were less than 5% significantly different from the control) in primary renal proximal tubule cells. This inconsistency of results may be due to different experimental conditions.

Insulin and IGF-I are antiphosphaturic in vivo and this effect is due, in part, to increased Na^+-dependent phosphate transport across the luminal brush-border membrane of the proximal tubule(Abraham et al, 1990) or OK cells (Caverzasio & Bonjour, 1989). However, the intracellular mechanism is not understood. The stimulatory effect of insulin and IGF-I on phosphate uptake was only partially prevented by cycloheximide, suggesting that both protein synthesis-dependent and -independent mechanisms are involved in this response. The inhibition of phosphate uptake was also partially dependent on gene transcription because it was inhibited by actinomycin D. In OK cells, IGF-I increases selectively the Na-dependent transport of phosphate by a mechanism involving the de novo synthesis of protein (Caverzasio & Bonjour, 1989). These data indicate that insulin and IGF-I increase Na/phosphate cotransport selectively through a mechanism that involves RNA and/or protein synthesis. These observations suggest that growth and growth hormone-related stimulation of renal phosphate transport could be mediated by IGF-I.

The second-messengers cAMP, diacylglycerol and inositol 1,4,5-triphosphate (IP_3) and Ca^{2+}($[Ca^{2+}]i$) have been implicated in Na/phosphate cotransport across the apical membrane of the proximal tubule (Quamme et al, 1994). The role of extracellular cAMP responsible for the stimulation of the transport of inorganic phosphate in response to insulin and IGFs were examined. Dibutyryl cyclic AMP increased the uptake of α-aminoisobutyric acid into purified rabbit renal brush border membranes. However the uptakes of α-methyl-D-glucoside, proline, leucine and phosphate into brush border membranes were significantly inhibited (Kippen et al, 1979). Exogenous addition of 8-bromo-cGMP (10^{-4} M) also significantly inhibited phosphate uptake by 14.6% in OK cells (Nakai et al, 1988). The inhibitory action of cAMP on brush border membrane phosphate transport is accompanied by stimulation of gluconeogenesis which suggests, indirectly, that changes in gluconeogenesis may be part of the intracellular mechanism for regulating brush border membrane phosphate uptake in response to certain stimuli (Kempson et al, 1983). In cultured OK cells, extracellular cAMP inhibited Na-dependent phosphate uptake in a time and concentration-dependent manner. The effect of cAMP was reproduced by ATP, AMP, and adenosine, and was blunted by dipyridamole which inhibits adenosine uptake. This report indicate that luminal degradation of cAMP into adenosine, followed by cellular uptake of the nucleoside by tubular cells, is the key event which accounts for the phosphaturic effect of exogenous cAMP (Friedlander et al, 1992). In cultured renal proximal tubule cells, extracellular cAMP ($1 \sim 100\ \mu M$) and forskolin (as a consequence of increase cAMP formation) inhibited phosphate uptake in a concentration-dependent manner. IBMX also inhibited phosphate uptake. These results suggest that insulin and IGF-I may act to decrease the levels of intracellular cAMP either through inhibition of adenylate cyclase activity or enhancement of phosphodiesterase activity.

The effect of heterotrimeric GTP-binding proteins on phosphate transport were also investigated. The infusion of cholera toxin, 4 ng/min, into one renal artery of normal and thyroparathyroidectomized dogs produced ipsilateral increments in the excretion

of Na, K, Ca, Mg and Cl. Phosphate excretion increased from both kidneys, but more from the infused kidney in intact dogs (Friedler et al, 1975). The effects of chorela toxin on electrolyte excretion could not be accounted for by changes in glomerular filtration rate or renal plasma flow. Urinary cAMP increased from both kidneys but slightly more from the infused kidney. This effect is probably mediated by the activation of renal adenylate cyclase sensitive to the enterotoxin. These observations demonstrate involvement of heterotrimeric G proteins in Na/phosphate cotransport. Thus, phosphate transport in renal proximal tubule cells seem to be regulated by Gs and Gi proteins, respectively.

PMA inhibited Na^+-dependent phosphate transport in fresh preparations of mouse renal tubules, elicited a rapid translocation of protein kinase C from the cytosolic to the particulate fraction and stimulated the phosphorylation of endogenous substrates in the cytosolic and brush border membrane fractions (Boneh et al, 1989). In the present investigations, we found that when protein kinase C was stimulated by PMA, DAG, and OAG, phosphate uptake in proximal tubule cells was inhibited. In LLC-PK_1 cells, activation of PKC with 12-O-tetradecanoylphorbol 13-acetate stimulated phosphate uptake (Mohrmann et al, 1986), but, in OK cells, phorbol-12, 13-dibutyrate and 1-oleoyl-2-acetyl-glycerol inhibited phosphate transport (Nakai et al, 1987). The paradoxical effects of PKC activation on phosphate transport may be a result of activation of different isoforms of PKC. It also possible that local stimulation of PKC due to specific receptor-mediated activation could have different effects than pharmacologically stimulating PKC with exogenous compounds. In the present study, blocking of PKC with staurosporine did not inhibited the effect of insulin to sitmulate phosphate transport. The present results suggest that PKC is one of the possible regulators in transducing the insulin and IGF-I signal for phosphate transport in primary cultured proximal tubule cells.

In conclusion, phosphate uptake is stimulated by high sodium and low phosphate and pH 6.5 in the culture medium. Membrane potential and intracellular energy levels are also an important factor for phosphate transport. Insulin and IGF-I stimulate phosphate uptake through a mechanisms that involve de novo protein and/or RNA synthesis and decrease of intracellular cAMP level. Also PKC is may play a regulatory role in transducing the insulin and IGF-I signal for phosphate transport in primary cultured proximal tubule cells.

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